Food Research

IN THIS ISSUE

The Evaluation of Processing Times for Raw-Pack Canning of Some Low-Acid Vegetables at Home

Effects of Three Levels of Nutrition and Age of Animal on the Quality of Beef. I. Palatability, Cooking Data, Moisture, Fat, and Nitrogen

Effects of Three Levels of Nutrition and Age of Animal on the Quality of Beef. II. Color, Total Iron Content, and pH

Effect of Three Levels of Nutrition and Age of Animal on the Quality of Beef. III. Vitamin B_{12} Content

Investigation of Copper Complexes Causing Cloudiness in Wines. I. Chemical Composition

Hydrolysis of Pectic Materials and Oligouronides by Tomato Polygalacturonase

Mechanism of Copper Casse Formation in White Table Wine. II. Turbidimetric and Other Physico-Chemical Considerations

Amino Acid Composition of Some Indian Vegetables as Determined by Paper Chromatography

Species of *Clostridium* Associated with Zapatera Spoilage of Olives

Strain Retardation in Starch Jelly Candy

Canning Experiments with Non-Sporeforming Bacteria

VOLUME 21, NUMBER 4 JULY-AUGUST, 1956

An Official Publication of

The Institute of Food Technologists

Food Research

An Official Publication of
The Institute of Food Technologists

Volume 21 JULY-AUGUST, 1956 Number 4

EDITORIAL BOARD

MARTIN S. PETERSON, *Editor-in-Chief*
(Editorial Office: 11606 South Bell Avenue,
Chicago 43, Illinois)

FRED E. DEATHERAGE
D. M. DOTY
FRANK L. GUNDERSON
MATHEW E. HIGHLANDS
ANDREA MACKEY
R. T. MILNER
LOUIS B. ROCKLAND
H. L. A. TARR
REESE H. VAUGHN
JAMES RICHARD VICKERY
R. O. WAGENAAR
O. B. WILLIAMS

FRED W. TANNER, *Editor Emeritus*
Z. I. KERTESZ, *Consulting Editor*

CHARLES S. LAWRENCE, *Business Manager*
176 West Adams Street
Chicago 3, Illinois

Copyright, 1956
By the Institute of Food Technologists
Printed in U. S. A.

Published by the Institute of Food Technologists six times a year. Printed by The Garrard Press, 119-123 West Park Avenue, Champaign, Illinois. Entered as second-class matter at the Post Office at Champaign, Illinois, under the act of March 3, 1879. Accepted for mailing at special rate of postage provided for in amended Section 34.40(e), pursuant to the provisions of Public Law 233, approved October 8, 1951.

Food Technology

Published Monthly by the

INSTITUTE OF FOOD TECHNOLOGISTS

The Companion Journal to

FOOD RESEARCH

If you are a reader of FOOD RESEARCH your library is incomplete without FOOD TECHNOLOGY—devoted to the publication of the results of research and their practical application in industry. Contributors to its pages represent many fields of scientific and technological interest.

SUBSCRIPTION RATES FOR 1956

FOOD RESEARCH	$8.50 Domestic; $10.00 Foreign
FOOD TECHNOLOGY . . .	$8.50 Domestic; $10.00 Canada, Cuba, and Mexico; $12.00 all other foreign.

Send Subscriptions to

FOOD TECHNOLOGY

THE GARRARD PRESS

119-123 West Park Avenue

CHAMPAIGN, ILLINOIS

Volume 21 JULY-AUGUST, 1956 Number 4

CONTENTS

THE EVALUATION OF PROCESSING TIMES FOR RAW-PACK CANNING OF SOME LOW-ACID VEGETABLES AT HOME. Sylvia Cover, Myrtis C. Shrode, and Howard Reynolds 405

EFFECTS OF THREE LEVELS OF NUTRITION AND AGE OF ANIMAL ON THE QUALITY OF BEEF. I. PALATABILITY, COOKING DATA, MOISTURE, FAT, AND NITROGEN. Marion Jacobson and Faith Fenton 415

EFFECTS OF THREE LEVELS OF NUTRITION AND AGE OF ANIMAL ON THE QUALITY OF BEEF. II. COLOR, TOTAL IRON CONTENT, AND pH. Marion Jacobson and Faith Fenton 427

EFFECT OF THREE LEVELS OF NUTRITION AND AGE OF ANIMAL ON THE QUALITY OF BEEF. III. VITAMIN B_{12} CONTENT. Marion Jacobson and Faith Fenton . . . 436

INVESTIGATION OF COPPER COMPLEXES CAUSING CLOUDINESS IN WINES. I. CHEMICAL COMPOSITION. C. E. Kean and G. L. Marsh 441

HYDROLYSIS OF PECTIC MATERIALS AND OLIGOURONIDES BY TOMATO POLYGALACTURONASE. B. S. Luh, S. J. Leonard, and H. J. Phaff 448

MECHANISM OF COPPER CASSE FORMATION IN WHITE TABLE WINE. II. TURBIDIMETRIC AND OTHER PHYSICO-CHEMICAL CONSIDERATIONS. A. Lukton and M. A. Joslyn 456

AMINO ACID COMPOSITION OF SOME INDIAN VEGETABLES AS DETERMINED BY PAPER CHROMATOGRAPHY. S. Ghosh Majumder, Rabindra Nath Dutta, and N. C. Ganguli 477

SPECIES OF *Clostridium* ASSOCIATED WITH ZAPATERA SPOILAGE OF OLIVES. Toshio Kawatomari and Reese H. Vaughn 481

STRAIN RETARDATION IN STARCH JELLY CANDY. Clarence Sterling 491

CANNING EXPERIMENTS WITH NON-SPOREFORMING BACTERIA. O. B. Williams . . 502

SUBSCRIPTION RATES

To members of the Institute of Food Technologists, $7.50 per volume.
Non-members: Domestic, $8.50 per volume; foreign, $10.00 per volume.
Subscriptions and renewals are entered to begin with the first issue of the current volume.
Single copies of current volume: Domestic, $1.50; foreign, $1.75.

CORRESPONDENCE

Editorial: MARTIN S. PETERSON, Editor-in-Chief, 11606 South Bell Ave., Chicago 43, Ill.
Non-Member Subscriptions, Back Volumes, Single Copies, and Reprints: FOOD RESEARCH, 119-123 W. Park Ave., Champaign, Ill.
Members Subscriptions: THE INSTITUTE OF FOOD TECHNOLOGISTS, 176 W. Adams St., Chicago 3, Ill.
Advertising: Charles S. Lawrence, Business Manager, 176 W. Adams St., Chicago, Ill.

CLAIMS

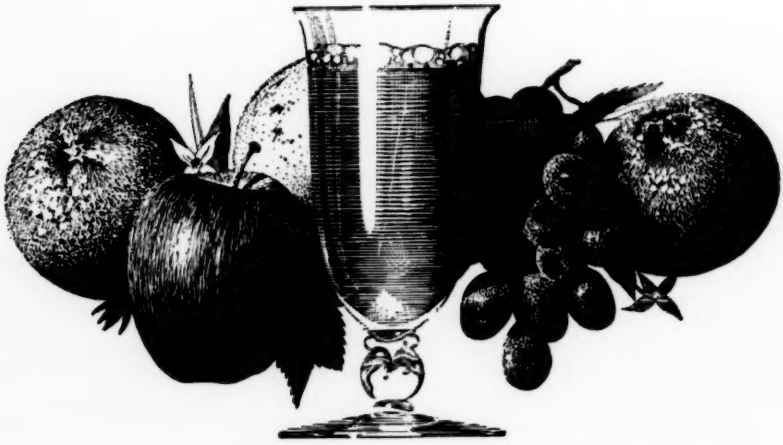
Claims for copies lost in the mails must be received within 30 days (90 days foreign) of the date of issue. Notice of change of address must be received two weeks before date of issue.

NOTE

The Institute of Food Technologists assumes no responsibility for statements in articles appearing in FOOD RESEARCH. The opinions expressed do not necessarily reflect policies of the Institute of Food Technologists.

Manuscripts for FOOD RESEARCH should conform to the style used in the journal and be submitted in duplicate to the Editor-in-Chief. Authors of papers intended for FOOD RESEARCH should request from the Editorial Office a copy of the booklet "Information and suggestions for authors." FOOD RESEARCH reserves the privilege of editing manuscripts to make them conform with the adopted style of the journal or returning them to the author for revision. Changes made by the Editorial Office may be reviewed by authors when they examine proof.

Juices need this advantage



Did you know that ascorbic acid may be added to certain fruit and vegetable juices to standardize their vitamin C content, thus overcoming wide variations which can occur ***(see table below)***. Many nutritionists believe that the public interest is best served when the ascorbic acid content of processed juices is so standardized. A label statement of 100% of the minimum daily adult requirement may be made when a juice serving contains 30 mg. of ascorbic acid.

When a food processor improves his juice by raising or standardizing its vitamin C content he merits your support. If you wish information about good juices which are now made better through the use of Roche ascorbic acid, please write to the Vitamin Division.

Ascorbic Acid Content of Canned Juices

Below are examples of minimum and maximum levels of ascorbic acid in commercially canned juices. Note the wide variations! All figures are in milligrams per 100 grams of juice. Data from U. S. Department of Agriculture.

	Min.	Max.
Grapefruit juice	10.0	49.0
Orange juice	9.7	70.0
Pineapple juice	5.4	18.0
Apple juice	0.2	3.6
Grape juice	0.0	4.7
Tomato juice	2.5	32.0

It is in the public interest to standardize the vitamin C content of these processed juices and juice products.

Orange — Grapefruit — Lemon — Tangerine — Apple Grape — Pineapple — Cranberry — Tomato Vegetable blends

No matter which type of processing is used— Canning • Concentrating • Freezing • Dilution in the form of "ades"—your juice will be better when its ascorbic acid content is standardized.

keeping faith with nature

ROCHE
ASCORBIC ACID

VITAMIN DIVISION • **HOFFMANN-LA ROCHE INC.** • NUTLEY 10, N. J.
Pacific Coast: L. H. Butcher Company, Los Angeles, San Francisco, Seattle, Portland, Salt Lake City
In Canada: Hoffmann-La Roche Ltd., Montreal, Quebec

THE EVALUATION OF PROCESSING TIMES FOR RAW-PACK CANNING OF SOME LOW-ACID VEGETABLES AT HOME

SYLVIA COVER
Texas Agricultural Experiment Station, College Station, Texas
MYRTIS C. SHRODE
Agent of the Human Nutrition Research Branch, College Station, Texas
AND
HOWARD REYNOLDS
Human Nutrition Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C.

(Manuscript received July 16, 1955)

The development of processing times for the home-canning of preheated packs of low-acid vegetables was initiated at a time when home-canning specialists generally favored preheating before packing to provide for more solid packs and better utilization of space in jars *(1, 3, 12)*. Processing studies on raw-pack canning of black-eye peas were reported in 1948 by Cover *et al. (1)*. Similar studies on snap beans have been reported by Dawson *et al. (2)*. The latter workers reported better quality in snap beans that were raw-packed than in those that were preheated before packing.

The raw-pack method of canning is easier and more convenient. The vegetables are handled less, fewer utensils are used, the area of surface required for cooking is less, and the total working time is shorter. Such important considerations from the viewpoint of the homemaker made work on other vegetables desirable.

EXPERIMENTAL PROCEDURE

The vegetables processed in this study were: green lima beans, carrots, cream style corn, whole kernel corn, English peas, and summer squash. Lima beans and peas were shelled and washed before packing; carrots were washed, scraped and sliced or diced; summer squash were washed, cut into ½-inch slices, and the latter halved or quartered to make pieces of uniform size. Corn for whole kernel packs was cut from the cobs at about two-thirds kernel depth. For cream style the cut was at about one-half kernel depth, the remaining portions being then scraped from the cob and mixed with the cut kernels before packing.

The clean prepared raw vegetables were weighed and packed directly into the jars. Boiling water was added until it was level with the top of each jar, the lid containing the thermocouple was adjusted, the jars placed in the pressure canner and the food processed at 10-pounds pressure (240° F.). The weight of the vegetable packed into the jars and the measurement of the head space before processing are given in Table 1.

Canning conditions for all of the raw packs were standardized in certain ways to simulate those conditions in the home which would have the most adverse effect on sterilizing values provided by a given process. The vegetables were chilled before packing, which lowered the sealing temperatures. A large gas burner was used to shorten the time needed to reach processing pressure. During heating of the pressure canner the windows and doors were closed and no fans were on. These practices insured lower "initial temperatures" than would have been possible otherwise. ("Initial temperature" is the temperature inside the jar when the pressure canner reaches 240° F., the processing temperature.) The cooling of the pressure canner to 212° F. was done in a draft—doors and windows open and fans on. Thus cooling time was reduced to a minimum. While these practices probably reduced the variation in process times, it is

TABLE 1

Weight of vegetable packed for canning

Vegetable	Weight of vegetable packed		Distance from top of jar to top of raw vegetable[1]	
	Pint jars	Quart jars	Pint jars	Quart jars
	grams	*grams*	*inches*	*inches*
Beans, fresh lima	245	515	1, (¾)	1-½, (1-¼)
Carrots	270	570	1	1
Corn, cream style	325	.....	1-½	.....
whole kernel	265	535	1	1-¾
Peas, English	250	540	1, (½)	1-½, (1)
Squash, summer	260	565	1	1

[1] The distances shown in parentheses are for the large type of vegetable—Fordhook limas and giant peas.

believed that they tended to lengthen the estimated process times and thereby to increase the margin of safety.

Processing times for the raw-pack vegetables were determined by procedures essentially as described by Toepfer *et al. (12)* and by Cover *et al. (1)*, with exceptions to be noted later in the method of estimating process values from heat penetration data.

Temperatures during processing were obtained by means of copper constantin thermocouples attached to a recording potentiometer as described previously, Cover *et al. (1)*. With cream style corn, a product heating essentially by conduction, thermocouple junctions were located at jar centers. With the other products, the junction was located on the longitudinal axis of the jar at ⅝ to 1 inch from the bottom. While "cold point" determinations were not made in these studies, the results of other investigations, Jackson *(5)*, Esselen and Tischer *(4)*, Townsend *et al. (13)*, and Desrosier and Esselen *(3)*, have shown this point to be near the geometrical center of the container with conduction heating products and on the longitudinal axis near the bottom when convection becomes significant. It is more correctly designated as an area of slowest heating rather than a point and, as noted by Townsend *et al. (13)*, variations of as much as ½ inch in thermocouple location on the longitudinal axis appeared to make little difference in observed heat penetration data, even in such homogeneous media as bentonite suspensions.

RESULTS AND DISCUSSION

Calculation of process values from heat penetration data were made by two methods depending on what data were used for statistical analyses —F_o values of individual jars or temperature readings for individual jars.

The processes for cream style corn were estimated according to the first method which has been described in detail by Toepfer *et al (12)*. A lethality curve was constructed for each jar. Areas under each curve corresponding to 3 or 4 process times were measured with a planimeter. These data were used to calculate the equation, $y_r = a + bx$, for regression of process value on process time, where x equals process time; y, the process value; a, the constant locating the process value–process time curve vertically; and b, the slope of the line. The constants in this equation for cream style corn in pint jars are $a = -16.846$, $b = 0.339$. The standard error of estimate is 1.106. From the regression equation and 2.6 times its standard error of estimate, process (F_o) values were estimated for each of three process times such that, on the basis of the distribution of observed data, the predicted probability of individual containers yielding lower F_o values is less than one in two hundred. The temperature data and calculated total process values are given in Table 2, along with those for the other vegetables studied.

TABLE 2

Temperature data and calculated minimum process values for raw-packed vegetables processed at 240° F. in pint and quart glass jars

Food	Container		Sealing temperature	Initial temperature[1]	Calculated minimum process values			
	Size	Number			Process times	Heating	Cooling	Total
			° *F.*	° *F.*	*mins.*	F_h	F_c	F_c
Beans, lima	Pints	12	130.2	221.1	16	2.20	1.55	3.75
					28	5.10	1.85	6.95
					36	7.33	1.85	9.18
	Quarts	12	124.5	211.3	27	3.14	1.32	4.46
					32	4.26	1.82	6.07
					40	6.10	1.53	7.63
					56	9.87	2.27	12.14
Carrots	Pints	12	117.3	219.1	13	1.29	0.95	2.24
					20	2.73	1.36	4.09
					32	5.58	1.60	7.18
	Quarts	9	109.2	210.4	30	3.40	1.60	5.00
					32	3.68	1.81	5.69
					48	7.69	2.16	9.85
Corn, cream style	Pints	12	121.0	152.1	80	.....	.....	7.39
					90	.....	.....	10.78
					100	.....	.....	14.17
whole kernel	Pints	12	111.2	202.4	37	3.74	1.19	4.93
					48	6.30	1.68	7.98
					60	9.61	1.68	11.29
					66	11.28	1.68	12.96
whole kernel	Quarts	13	108.4	187.5	53	3.73	0.96	4.69
					78	9.36	1.62	10.98
					84	10.70	2.12	12.82
Peas, English	Pints	11	124.7	220.8	25	3.37	1.05	4.42
					40	6.60	1.48	8.08
					52	9.41	1.75	11.16
	Quarts	12	116.7	212.1	20	1.99	0.93	2.92
					40	6.54	1.15	7.69
					60	11.20	1.66	12.86
Squash, summer	Pints	12	112.9	218.4	10	0.64	0.73	1.37
					20	2.55	1.44	3.99
					24	3.53	1.44	4.97
					40	7.30	1.78	9.08
	Quarts	12	110.3	208.9	26	2.53	1.17	3.70
					33	4.02	1.39	5.41
					44	6.64	2.42	9.06

[1] Jar temperature at time when pressure cooker reached 240° F.

Initial temperatures in Table 2 are the jar temperatures at the time when the pressure cooker reached processing temperature. Since the come-up time with household pressure canners is 10 to 15 minutes and this is preceded by a 10-minute exhaust, there is normally a significant increase

in jar temperatures during this 20- to 25-minute period and initial temperatures, so designated, will often be in excess of 212° F., especially with rapid heating products.

Results reported by Toepfer *et al. (12)* showing that process values estimated from replicated heat penetration determinations on a specific product are normally distributed, permit application of the above outlined statistical procedure to the estimation of limits within which process values may be expected to fall. Furthermore, the reliability of such estimates was confirmed by inoculated packs. It is pertinent to note also that process times calculated by Desrosier and Esselen *(3)* using this method were of the same order of magnitude but generally somewhat longer than when estimated from a composite of the slowest heating and the fastest cooling data from a group of replicated heat penetration determinations.

For vegetables other than cream style corn the statistical treatment of the data was made on the jar temperatures instead of on the process values so that planimeter measurements were required on only one lethality curve for each vegetable and size of jar. Jar temperatures were recorded at 4-minute intervals from the time the pressure canner reached 240° F. Temperatures were averaged for each 4-minute interval, and the means minus 2.6 times their standard deviations were used for calculating the corresponding lethal rates for $z = 18$. These lethal rates were used to construct only one lethality curve for each food and size of jar, measurements of the area were made at three process times and the results are given in Table 2.

The procedure is illustrated with the data for lima beans in quart jars. In Table 3 are given the mean temperatures at 4-minute intervals during processing at 240° F. and during cooling, the statistical computations for these temperatures and the corresponding lethal rates. On the basis of the distribution of the observed temperature data, lethal rates lower than those in the last column of Table 3 would be expected to occur less than once in two hundred determinations with comparable packs of lima beans. These lethal rates were used to construct one lethality curve by plotting lethal value against time on coordinate paper. The area under the lethality curve was measured with a planimeter. Construction of the lethality curve, and measuring the area under it so that the area in inches would also represent the total lethal value (F_o) of the heating and cooling periods have been illustrated and described in detail by Toepfer *et al. (12)* and Cover *et al. (1)*.

An adequate process is one yielding an F_o equivalent to the F value of the spoilage organism which the process is designed to destroy. Process times for all of the products studied were designed to yield sterilizing values which would be equivalent to those used by Toepfer *et al. (12)* for preheated-packs of low-acid foods. To determine these times, the three or four process values (F_o) for each product in Table 2 were plotted against their corresponding process times. The times required to provide the proposed sterilizing values were then read from the resulting process value–process time curves. The results are given in Table 4. Included in Table 4 for comparison are the estimated process times reported by Toepfer *et al. (12)* for preheated-packs of the same vegetables. Determination of the

TABLE 3

Jar temperatures and estimated minimum lethal rates during processing at 240° F. and cooling of lima beans in quart jars

Processing time	Mean jar temperature (12 jars)	Standard deviation	2.6 × standard deviation	Mean temperature—(2.6 × standard deviation) and corresponding lethal rates for z = 18	
				Temperature	Lethal rate
minutes	*° F.*	*° F.*	*° F.*	*° F.*	*F/t.*
–4	194.5	2.93	7.6	186.9	0.0003122
0	211.3	2.01	5.2	206.1	0.003640
4	223.7	1.11	2.9	220.8	0.02387
8	230.5	0.50	1.3	229.2	0.06989
12	234.4	0.49	1.3	233.1	0.1151
16	236.6	0.49	1.3	235.3	0.1525
20	237.6	0.49	1.3	236.3	0.1733
24	238.2	0.37	1.0	237.2	0.1945
28	238.7	0.47	1.2	237.5	0.2021
32	239.0	.....	....	239.0	0.2449
36	239.3	0.43	1.1	238.2	0.2210
40	239.3	0.43	1.1	238.2	0.2210
44	239.5	0.50	1.3	238.2	0.2210
48	239.7	0.47	1.2	238.5	0.2297
52	239.8	0.37	1.0	238.8	0.2387
56	240.0	.....	....	240.0	0.2782
60	239.3	0.72	1.9	237.4	0.1995
64	237.3	1.88	4.9	232.4	0.1052
68	234.0	2.52	6.6	227.4	0.05551
72	231.1	3.20	8.3	222.8	0.03082
76	227.9	3.95	10.3	217.6	0.01585
80	221.6	4.63	12.0	209.6	0.005696
84	214.7	5.01	13.0	201.7	0.002073
88	207.6	5.06	13.2	194.4	0.0008149
92	201.4	4.86	12.6	188.8	0.0003981

minimum process times estimated to yield process values high enough for safety are illustrated with the data on lima beans. The four process values (F_o) from Table 2 for quart jars of lima beans were plotted against their corresponding process times (Figure 1A). The processing time for quart jars of lima beans was read from this curve at the point where an F_o of 9.3 intercepts the curve. It was 45.0 minutes.

For purposes of comparison, three additional methods of treating the heat penetration data for quart packs of lima beans were used for deriving estimates of processing times at 240° F. expected to yield an F_o value of 9.3. In one, measurements were made of areas under individual lethality curves for each of the 12 jars. The resulting F_o values were treated as described previously for cream style corn. The constants of the equation for regression of process value on process time were found to be, a = –2.344, b = 0.293. The standard error of estimate is 0.542. From the regression equation defined by the above constants and 2.6 times its standard error of estimate, the process time expected to yield a minimum F_o value of 9.3 minutes was estimated to be 44.5 minutes. This estimate is in excellent agreement with the 45.0-minute process derived by the method first described. A third estimate of the time required to yield a minimum F_o value of 9.3 was

TABLE 4

Process times at 240° F. estimated to yield process values that are equivalent to or greater than the suggested process requirements for raw-packed vegeables

Food	Suggested process requirement	Container size	Calculated processing time at 240° F.	
			Raw-packed	Preheated[1]
	F_o		*minutes*	*minutes*
Beans, lima	9.3	Pints	37	32
		Quarts	45	57
Carrots	5.0	Pints	24	19
		Quarts	30	25
Corn, cream style	12.8	Pints	96	83
Corn, whole kernel	12.8	Pints	66	54
		Quarts	84	82
Peas, English	8.5	Pints	41	38
		Quarts	43	41
Squash, summer	5.0	Pints	24	28
		Quarts	31	37

[1] From Toepfer *et al. (12).*

derived from the same data by measurements of a lethality curve representing a composite of temperature data from the fastest heating–slowest cooling jars. From this curve the F_o values at 30, 40, and 50 minutes were 5.3, 8.2, and 10.6, respectively. From the process value–process time curve defined by these three points, the processing time required to yield an F_o value of 9.3 is estimated as 44.5 minutes. Thus, the three methods described

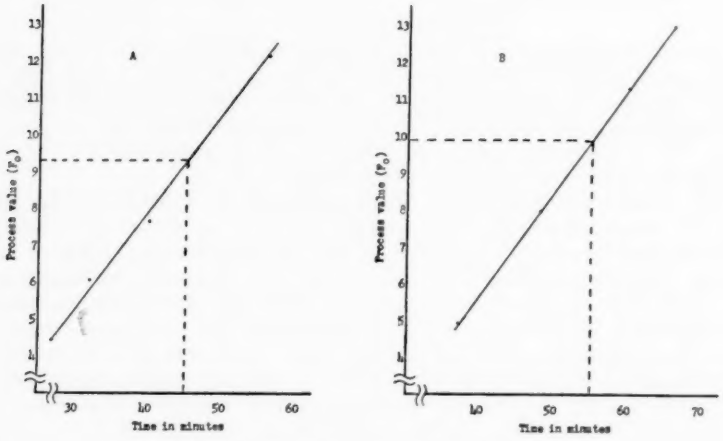


Figure 1. Process value—process time curves.
A. Lima beans in quart jars.
B. Whole kernel corn in pint jars.

for evaluating heat penetration data have provided estimates of the process time at 240 required to yield a minimum F_0 value of 9.3 during the processing of quart packs of lima beans which differ by only 0.5 minute. A fourth estimate was based on the unadjusted average process value–process time curve. From this curve, the F_0 values were 6.5, 9.4, and 12.3 for processing times of 30, 40, and 50 minutes, respectively, and the time required to yield an F_0 value of 9.3 was estimated as 40 minutes. This is 5 minutes shorter than when statistical limits were calculated and used to estimate process times expected to yield the required process as a minimum rather than as an average.

The calculated process times for raw-packs (Table 4) may be expected to meet or exceed the suggested process requirements in at least 199 out of 200 similarly prepared and processed containers and are generally of the same order of magnitude as those determined by Toepfer *et al. (12)* with preheated-packs. The previously noted studies of Cover *et al. (1)* and Dawson *et al. (2)* have provided evidence that, with either preheated- or raw-packs of convection heating low-acid foods, similar processing times may be expected to yield comparable sterilizing values. Variations between the data for raw- and preheated-packs will be discussed in detail.

For lima beans, the raw-pack data led to the calculation of 37 and 45 minutes as estimates of processing times at 240° F. required to yield F_0 values of 9.3 or greater with pint and quart packs, respectively. These times vary somewhat from those determined for preheated-packs (Table 4). Examination of the two sets of primary data for quart jars showed that the difference could be largely attributed to the fact that heat penetration was considerably more variable in the preheated- than in the raw-packs, the respective standard errors with quart packs being 2.29 and 0.54. (The lower standard errors in the raw packs may have been caused by the standardization of procedure described under the method of canning.) Average lethal value–process time curves for quart jars of the two packs were in good agreement, however. For example, the mean F_0 values of the 50-minute process were 12.4 and 13.3 for raw- and preheated-packs, respectively. Comparability of the means permitted an over-all analysis of the two sets of data. From the combined data, F_0 values of 5.1, 8.1, and 11.0 were estimated as the minimum values to be expected during 40-, 50-, and 60-minute processes, respectively. From the process values–process time curve defined by these three points, 54 minutes was estimated as the process time at 240° F. which would be expected to yield an F_0 value of 9.3 minutes or greater in not less than 199 of 200 jars of either raw or preheated quart packs of lima beans. As noted above, these data also show that a process of 50 minutes would be expected to yield a minimum F_0 value of 8.1 minutes, which is slightly greater than sterilizing values yielded by commonly used commercial processes for lima beans in containers of comparable size. On the basis of the data for raw-packed lima beans, a process of 40 minutes for pint jars would be expected to yield F_0 values of at least 9.3 or higher.

Process times for raw-packed carrots are somewhat longer than those estimated to yield similar F_0 values with preheated-packs. As with the lima beans, these data also show no significant difference in the mean rates of heat penetration during the processing of raw- and preheated-packs.

Similarity of process requirements for these packs would be expected, moreover, since the short heating (bringing to boil) applied in preparing the preheated-packs does not alter greatly the texture or the weight of carrots packed into jars of comparable size. On the basis of the data for raw-packed carrots, processes of 24 and 30 minutes for pints and quarts, respectively, would be expected to yield F_0 values of at least 5.0 or higher.

With products such as cream style corn, in which heat transfer is largely by conduction, low initial temperatures tend to cause significant increases in processing times required to yield specified sterilizing values. The longer process for raw-packs than for the preheated-packs of cream style corn is attributed to an average difference of 40° F. in initial temperatures of these packs. Thus, the raw-packs would require 95 minutes of processing at 240° F. and the preheated-packs 85 minutes, on the assumption that for this product a safe process should yield an F_0 value of 12.8.

With raw-packs of whole kernel corn in quart jars, a process of 85 minutes was estimated to yield an F_0 value of 12.8 or higher. This process time for raw-packs is in excellent agreement with that previously reported for preheated-packs of whole kernel corn (Table 4). Since factors other than container size which affect heat penetration were the same, we should also expect that a given process for pints would yield similar sterilizing values with both types of pack and there is no obvious explanation for the observed difference between pints of the two packs. In connection with processes for corn it should be noted that the selected process (F_0) value of 12.8 is probably somewhat greater than is required for safety and sterility. This suggestion finds support in thermal-death-time data now available. Studies by Sognefest *et al. (10)*, Reed *et al. (8)*, and Reynolds *et al. (9)* have shown that the maxmium resistance of P.A. 3679 spores when heated in canned corn is in the range of 7 to 10 minutes at 250° F. Thus, a process for corn with an F_0 value of somewhat less than 10 minutes is adequate to destroy bacterial spores of comparable heat resistance to those of P.A. 3679 and provide a considerable margin of safety with respect to the destruction of *Clostridium botulinum* spores. On the basis of the heat penetration data reported here for raw-packed whole kernel corn processed in pint jars, the estimated minimum lethal value (F_0) of a 55-minute process would be 9.9 minutes (Figure 1B) which is approximately one minute in excess of F_0 values yielded by commercially-used canning processes for whole kernel corn in containers of comparable size. In this connection also, it is pertinent to note that a process of 50 minutes at 240° F. for whole kernel corn packed without preheating in 303 × 411 glass jars (approximately 1-pint capacity) is recommended for commercial packers, National Canners Association *(6)*. Because the latter process is followed by spray or water cooling, the cooling period would contribute less to the total process lethality than does the slow cooling of glass jars in home-canning procedures.

With peas, the agreement between the processes of 41 and 43 minutes calculated for pints and quarts from raw-pack data and the previously estimated 38 and 40 minutes for preheated-packs is sufficiently close to permit consideration of these as check determinations.

With summer squash, the data show that heat penetration during processing was definitely more rapid in the raw- than it was in the preheated-

packs. This difference is attributed to the fact that even the short heating of this product for preheated-packs caused sufficient softening of the squash, so that these packs were considerably more solid than the raw-packs. With this product, therefore, the indicated processes required to yield a minimum F_o value of 5.0 is somewhat shorter for raw- than for preheated-packs. Processing times of 25 and 30 minutes for pints and quarts, respectively, are indicated for raw-packs of summer squash, but with the preheated-packs the somewhat longer processes of 30 and 35 minutes, respectively, still seem indicated.

That the processes indicated by the data in this investigation have adequate margins of safety for use in home-canning procedures is supported by several considerations. Extensive data on heat penetration and inoculated pack studies presented by Toepfer *et al. (12)* provide substantial evidence that processes derived in this manner, to yield the selected sterilizing (F_o) values for these vegetables, are adequate to sterilize the products in question with respect to spores of putrefactive anaerobe, P.A. 3679. These authors, as well as Pilcher *(7)*, and Stumbo *(11)*, have pointed out the considerable margin of safety with respect to *Clostridium botulinum* that is inherent in processes designed to achieve sterility with respect to an organism exhibiting the heat resistance of P.A. 3679. In addition, the F_o values yielded by the indicated processes are in all cases higher than F_o values yielded by commonly-used commercial processes for these products in containers of comparable sizes. For example, commercial processes in 2-½ and smaller-sized cans yield F_o values of 3.0 to 3.5 for carrots and 8 to 9 for whole kernel corn, as compared with the F_o values of 5 and 12.8, respectively, yielded by the indicated processes reported here for these products in quart and pint jars.

SUMMARY

Process times have been evaluated for raw-pack canning of some low-acid vegetables at home. Heat penetration data from the raw-pack studies have been compared with previously reported data on preheated-packs.

LITERATURE CITED

1. Cover, Sylvia, Smith, A. B., Sheppard, M. P., and Crawford, E. G. Development of safe methods for canning black-eye peas at home. Texas Agr. Expt. Sta., *Bull. 707*. (1948).
2. Dawson, E. H., Gilpin, G. L., Warren, H. W., and Toepfer, E. W. Canning snap beans: precooked and raw-pack. *J. Home Ec.*, **45**, 165-168 (1953).
3. Desrosier, N. W., and Esselen, W. B., Jr. Heat penetration and processing studies on home-canned corn, hominy, pork and beans, and potatoes. Mass. Agr. Expt. Sta., *Bull. 456*. (1950).
4. Esselen, W. B., Jr., and Tischer, R. G. Home canning. II. Determination of process times for home-canned foods. *Food Research*, **10**, 215-226 (1945).
5. Jackson, J. M. Mechanisms of heat transfer in canned foods during thermal processing. *Proc. Institute of Food Technologists*. 39-50 (1940).
6. National Canners Association. Processes for low-acid foods in glass containers. *Bull. 30-L*, 2nd ed., (1948).
7. Pilcher, R. W. Modern thermal process determination. *The Canned Food Reference Manual*, 3rd ed., 1949, American Can Co., New York, 297-321.
8. Reed, J. M., Bohrer, C. W., and Cameron, E. J. Spore destruction-rate studies on organisms of significance in the processing of canned foods. *Food Research*, **16**, 383-408 (1951).

9. Reynolds, H., Kaplan, A. M., Spencer, F. B., and Lichtenstein, H. Thermal destruction of Cameron's putrefactive anaerobe 3679 in food substrates. *Food Research*, 17, 153-167 (1952).
10. Sognefest, P., Hays, G. L., Wheaton, E., and Benjamin, H. A. Effect of pH on thermal process requirements of canned foods. *Food Research*, 13, 400-416 (1948).
11. Stumbo, C. R. Thermobacteriology as applied to food poisoning. *Advances in Food Research*, II, Academic Press Inc., New York, 47-115 (1949).
12. Toepfer, E. W., Reynolds, H., Gilpin, G. L., and Taube, K. Home-canning processes for low-acid foods developed on the basis of heat penetration and inoculated packs. U.S.D.A. *Tech. Bull. 930*. (1946).
13. Townsend, C. T., Reed, J. M., McConnell, J., Powers, M. J., Esselen, W. B., Jr., Somers, I. I., Dwyer, J. J., and Ball, C. O. Comparative heat penetration studies on jars and cans. *Food Technol.*, 3, 213-226 (1949).

EFFECTS OF THREE LEVELS OF NUTRITION AND AGE OF ANIMAL ON THE QUALITY OF BEEF. I. PALATABILITY, COOKING DATA, MOISTURE, FAT, AND NITROGEN

MARION JACOBSON[a] AND FAITH FENTON
State College of Home Economics and the State Agricultural Experiment Station, Cornell University, Ithaca, New York

(Manuscript received September 8, 1955)

This study was made to determine the effects of three feeding levels upon the palatability and nutritive value of beef. The influence of age of the animal upon palatability factors and nutritive value were also observed. Opportunity to measure these effects arose from a project being carried out by the Department of Animal Husbandry, New York College of Agriculture, on the influence of three levels of nutrition on the growth and development of Holstein bulls and heifers. Development of the reproductive organs and endocrine glands of the animals has been described by Musgrave *(8)*, Dunn *(3)*, and Sorensen *(10)*; data on the whole body composition and carcass characteristics have been reported by Wellington *(11)*.

Roasts prepared from isolated muscles from these animals of controlled history were the basis of this investigation. Since approximately one-third of the beef used in the United States in 1953 came from dairy breed animals,[b] it was thought that a study of the palatability and nutritive value of beef from this type of animal would be of value.

History of the meat. Three stripped-out muscles *(longissimus dorsi, psoas major, and semimembranosus* with *adductor)* were obtained from each of 24 heifer carcasses. There were duplicate animals at each level of nutrition (low, medium, high) for each age (32, 48, 64, 80 weeks).

Levels of Nutrition. Feeding was controlled on the basis of the T.D.N. (total digestible nutrients) intake. The 3 levels of intake represented 60%, 100%, and 160% of the amounts of total digestible nutrients according to the upper limits of Morrison's (1949) standards for growing dairy heifers. Amounts of milk and the quality of the hay fed, as well as amounts of starter and grower, were adjusted to the levels of nutrition. Animals on the high feeding level also received a mineral supplement daily and a supplement of vitamins A and D weekly. Composition of the starter and grower, as well as the amounts of minerals (Fe, Mn, Cu, Co, Mg) added to the rations on the high level, were described by Dunn *(3)* and Sorensen *(10)*.

Aging of Carcass and Dissection of Muscles. After each carcass had been aged 7 days at 1-3° C. (33-37° F.) and at humidity of 80 to 90%,

[a] This is part of a study submitted in partial fulfillment of the requirements for the Ph.D. degree by Marion Jacobson. Present address: School of Home Economics, Purdue University, Lafayette, Indiana.

[b] Personal communication from A. V. Nordquist, Chief Livestock and Poultry Statistic Branch, Agricultural Marketing Service, U. S. Department of Agriculture.

the muscles were dissected from the right side of each carcass. These muscles were kept under refrigeration until they could be prepared for freezing within a few hours.

Freezing and storage procedures. In preparation for freezing the stripped-out muscles were trimmed of exterior fat and membrane. Uniformly placed samples were removed for shear and color tests and for chemical and microbiological analyses. The remainder of each muscle was cut into 1 or 2 roasts, approximately 454 g. in weight and cylindrically shaped with dimensions of 6 inches (15 cm.) in length and 2-½ inches (6-½ cm.) in diameter. The length of the roast was cut parallel with the muscle fibers. The roasts were prepared in uniform size and shape to reduce variability in results owing to differences in cooking time. In preliminary work it had been noted that large roasts cooked to a stated internal temperature were more "done" than small roasts cooked to the same temperature.

Thermocouples were inserted in each roast prior to wrapping the meat in cellophane (Dupont MSAT-87). Roasts were frozen in a home-type freezer having an air temperature of approximately –23° C. (–9° F.) until the tests were made, 3 to 8 months later. Comparable samples had the same length of storage.

Diagrams of the sampling of each of the 3 muscles for roasts, objective tests, and chemical and microbiological analyses of the raw meat are given in Figure 1.

EXPERIMENTAL

Roasting method. Three roasts were cooked and tested in each trial. They were the same muscle from animals of the same age that had been fed at each of the 3 nutritive levels. Hard-frozen roasts were placed on ½-inch-high wire racks in open, round stainless steel pans. Roasting was done in a well-insulated, electric test oven with continuous recording of internal temperature by means of an electronic recording potentiometer. Roasts were removed from the oven when an interior temperature of 69° C. (156° F.), medium doneness, was indicated. Prior to sampling, each roast was allowed to cool to a constant temperature of 60° C. (140° F.), in order to control variability in drip during slicing.

Palatability tests. The remainder of the meat, after first removing samples for shear and color tests, was sliced to a constant thickness of ⅛-inch by use of a hand-operated slicer. From each slice to be used for tasting, a one-inch square was removed for the "chew" *(6)* test. Each judge received the square for "chew" testing which had been cut from the slice which he was given for evaluating the other palatability factors.

A panel of 6 or 7 trained judges, men and women, scored the cooked meat for color, aroma, juiciness, flavor, and tenderness, using a ten-point scale for each factor. Tenderness was also evaluated by the Lowe *(6)* "chew" method. The meat was at room temperature when it was judged.

Shear tests. Cores, 1 inch in diameter, were cut from thawed samples of the raw meat and from a one-inch slice taken from the center of each cooked roast. Each core was sheared once on the Warner-Bratzler shear apparatus.

Chemical analyses. The raw or cooked beef was ground and mixed twice in preparation for the chemical analyses. Moisture, fat, and total nitrogen of these samples were determined. Separate samples were analyzed for iron and vitamin B_{12} content; the results for these nutrients will be reported subsequently in separate papers.

Moisture: Five-gram samples of the ground raw or cooked meat were analyzed for moisture by the A.O.A.C. *(1)* method. Samples were dried in a vacuum oven for a constant time of 7 hours.

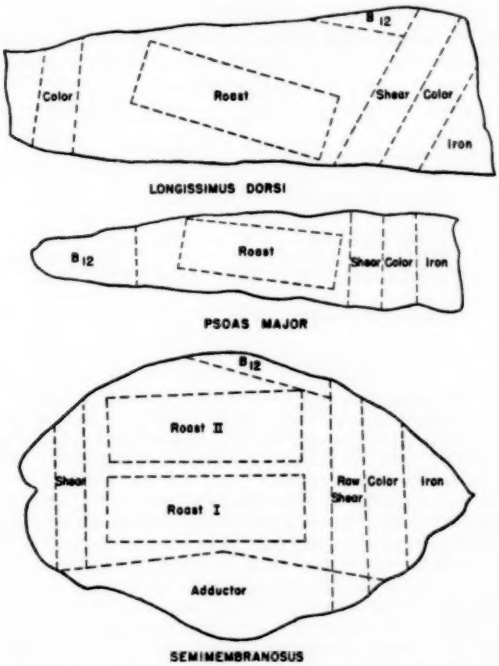


Figure 1. Diagrams of sampling of three uncooked muscles for one-pound roasts, objective tests, and chemical analyses, after being trimmed of exterior fat and membrane.

Fat: The Babcock method, adapted for meat from the A.O.A.C. method for milk, was used in the determination of the fat content of the ground raw and cooked samples.

Nitrogen: The method used for the assay of total nitrogen content of the raw beef was that of Ma and Zuzaga *(7)*; analyses of the cooked meat were not made.

Statistical methods. *Design:* A randomized block design was planned by Sorenson *(10)*. The 3 treatments involved animals on low, medium, or high level of nutrition. Animals were randomly selected for slaughter at the various ages. This study followed the design set up for the rearing of the animals.

Treatment: The statistical interpretation of the data was used on the method of analysis of variance of Snedecor *(9)*.

RESULTS

Raw meat. *Size of the Whole Muscles:* Weights of the muscles increased directly with the increase in the level of nutrition (Table 1). The increment owing to nutrition was statistically significant at the 1% level for all muscles at 32, 48, 64, and 80 weeks of age. Figure 2 illustrates how the size, and also the depth of color and extramuscular fat, varied in these stripped-out muscles with the level of nutrition. Comparison of the chilled carcass weights by Wellington *(11)* had also indicated a striking difference in total weight owing to feeding.

As expected, age was a highly significant factor influencing the weights of the muscles (Table 1).

Moisture: The consistent decrease in moisture content with increase in level of nutrition (Table 2) was highly significant for each of the 3 muscles tested from the 24 carcasses. A decrease in moisture content was evident with increase in age (Table 3).

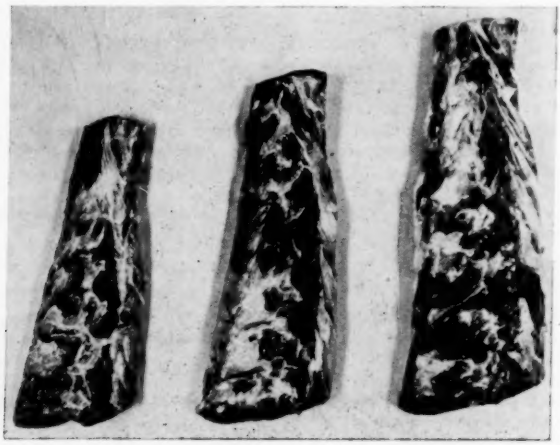
TABLE 1

Average weights of muscles from heifers reared on one of three planes of nutrition

Weeks of age	Muscle	Level of nutrition		
		Low	Medium	High
		g.	*g.*	*g.*
32	*Longissimus dorsi*	208	403	798
	Psoas major	210	336	602
	Semimembranosus	633	1409	2208
48	*Longissimus dorsi*	663	1174	1636
	Psoas major	402	697	943
	Semimembranosus	1780	3492	3691
64	*Longissimus dorsi*	858	1615	2038
	Psoas major	363	478	482
	Semimembranosus	2398	4106	4019
80	*Longissimus dorsi*	994	1836	2516
	Psoas major	388	654	772
	Semimembranosus	2903	4446	5258

These differences were significant at the 1% level for the *longissimus dorsi* and *psoas major* muscles, and at the 5% level for the *semimembranosus* (with *adductor*).

Fat: A stepwise increase in fat content with increase in level of feeding (Table 2) was observed for each of the 3 kinds of muscles and the differences were significant at the 1% level. An increase in the fat content was also noted with increase in age (Table 3). Differences were significant at the 1% level in the *longissimus dorsi* and *psoas major* muscles from animals on the medium and high planes of feeding and at the 5% level for the *semimembranosus* muscle. The fat in the muscles from animals on the low level of nutrition increased very little up to the highest age of 80 weeks.



Low Medium High

Figure 2. Photograph illustrating the variation in size, depth of color, extramuscular and intramuscular fat of the stripped-out longissimus dorsi muscles from 64 week Holstein heifers reared on low, medium, and high levels of nutrition.

TABLE 2

Effect of level of nutrition on the mean shear values and moisture, fat, nitrogen content of uncooked fat-trimmed muscles (longissimus dorsi, psoas major, and semimembranosus)[1]

Level of nutrition	Shear	Moisture	Fat	Nitrogen	
				Moist basis	Dry fat free
	lb.	%	%	%	%
Low	20	75.5	0.8	3.22	13.7
Medium	21	74.2	1.8	3.35	13.9
High	18	73.0	2.7	3.34	13.8

[1] Each value is the mean of 24-27 muscles (6 muscles at each age for each level).

Fat, Moisture: As the fat increased in the muscle the moisture decreased. This relationship was observed in all 3 muscles.

Nitrogen: The mean of the total nitrogen content on the moist basis in all of the muscles was 3.30%. There were no significant differences in total nitrogen content on the dry, fat-free basis that could be attributed to the levels of nutrition (Table 2). Differences in total nitrogen content on the dry, fat-free basis owing to age were highly significant for each of the 3 muscles. In the *semimembranosus* muscle these differences represented a small but consistent increase in total nitrogen with each age period, whereas in the other 2 muscles the increases were the greatest between 64 and 80 weeks of age. Mean values for these data are presented in Table 3.

Shear Force Values: Marked variability was observed among values for cores from adjacent parts of the same muscle and also between muscles from animals on the same feeding. There were no significant differences in shear values which could be attributed to the level of nutrition (Table 2). No consistent evidence was found that the shear force value was changed with the increased age of the animal up to 80 weeks (Table 3). Mean shear force value for the *psoas major* muscle as expected was lower than that for the *longissimus dorsi* and the *semimembranosus* at each age.

COOKED MEAT

Cooking data. *Thawing-roasting Times:* The range of mean thawing-roasting times from −12° C. (10° F.) to 69° C. (156° F.) for those roasts weighing from 440 to 460 g. was from 102 to 125 minutes. There were probably other factors than weight and dimensions affecting the thawing-roasting time, yet the variations in cooking time were markedly decreased when roasts were uniform in size and shape.

Weight Loss: Mean weight loss for all the roasts was 18.0 per cent. The per cent weight lost during cooking did not vary significantly with the level of nutrition of the animal (Table 4) or with the age of the animal (Table 5).

Drip Loss: Mean drip loss from all the roasts was 3.9%. The fat drip loss was negligible. Drip loss tended to be somewhat higher for roasts from animals on medium

TABLE 3

Effect of age of animal on the mean shear values and moisture, fat, nitrogen content of uncooked fat-trimmed muscles (longissimus dorsi, psoas major, and semimembranosus)[1]

Age	Shear	Moisture	Fat	Nitrogen	
				Moist basis	Dry fat free
weeks	*lb.*	%	%	%	%
32	22	75.4	1.2	3.19	13.3
48	19	74.2	1.3	3.25	13.3
64	19	74.1	2.0	3.28	13.8
80	22	73.4	2.6	3.51	14.7

[1] Each value is the mean of 18-24 muscles (6 muscles at each level for each age, with some additional replicates).

TABLE 4

Effect of level of nutrition on mean losses during roasting of beef[1]

Level of nutrition	Weight	Drip	Losses	
			Coagulum	Evaporation
	%	%	%	%
Low	17.3	3.2	2.1[2]	14.0
Medium	18.4	4.1	3.1	14.4
High	18.4	3.7	2.9	14.7

[1] Each value is the mean of 28-36 roasts.
[2] The coagulum was a part of the drip loss.

or high nutritive levels, the differences however were not statistically significant (Table 4). For roasts from the *semimembranosus* muscles the drip loss decreased regularly with increased age; means for the data for all the muscles did not show the relationship (Table 5).

Coagulum: The range of coagulum loss for roasts approximately 454 g. in weight was from 3 to 23 g., with a mean value of 11.6 g. The per cent range was from 0.9 to 8.4 with a means of 2.9%. Since the coagulum developed from the drip, these two losses closely parallel each other. Coagulum losses appeared to be slightly higher at the two highest levels of nutrition (Table 4). Coagulum losses showed a tendency to decrease with increase in the age of the animal (Table 5).

Evaporation Loss: The average loss owing to evaporation was 14.2%. No relationship was demonstrated between evaporation loss and level of nutrition of the animal or age of the animal between 32 and 80 weeks (Tables 4, 5).

Palatability. *Aroma:* Mean aroma scores on any one roast ranged from 5.7, *above average,* to 8.3, *very good. Level of nutrition:* No significant differences in aroma scores owing to level of nutrition occurred (Table 6). *Age:* A slight preference was indicated for the aroma of beef from animals slaughtered at 48 weeks of age (Table 7). Differences owing to age were mathematically significant for the *longissimus dorsi* and the *psoas major* muscles. These observations were of course subject to a possible variation of the panel at successive periods of judging. *Muscles:* Roasts from *semimembranosus* muscles tended to have slightly higher scores for aroma than did those from the *longissimus dorsi* and the *psoas major.* The scores for aroma were thus in the same order as the activity of the muscles.

Flavor of Lean: Mean flavor scores on any one roast ranged from 5.7, *average,* to 8.8, *almost excellent. Level of nutrition:* The flavor of the *longissimus dorsi* muscle from animals on the 2 higher levels of nutrition received significantly higher scores than did the same muscle from the low level of nutrition (Table 6). In the other muscles studied the same trend was noted. *Age:* The roasts from the animals 48 weeks of age received higher scores than did those from animals at the other ages (Table 7). No explanation is known for the flavor of roasts from the 48-week-old animals being scored higher than that of roasts from the 64- and 80-week-old animals. *Muscles:* No relationship was apparent between the particular muscles and the flavor scores of the lean.

Juiciness: Mean scores for juiciness of individual roasts ranged from 5.7, *average,* to 8.8, *almost excellent. Level of nutrition:* Although the *psoas major* muscles from the animals on the medium level of nutrition were judged significantly higher in juici-

TABLE 5

Effect of age of animal on mean losses during roasting of beef[1]

Age	Weight	Drip	Losses	
			Coagulum	Evaporation
weeks	%	%	%	%
32	19.6	6.1	5.4	13.4
48	16.4	3.5	2.7	12.9
64	16.8	3.4	2.3	13.5
80	19.8	3.5	2.4	16.2

[1] Each value is the mean of 4-12 roasts depending on level and age.

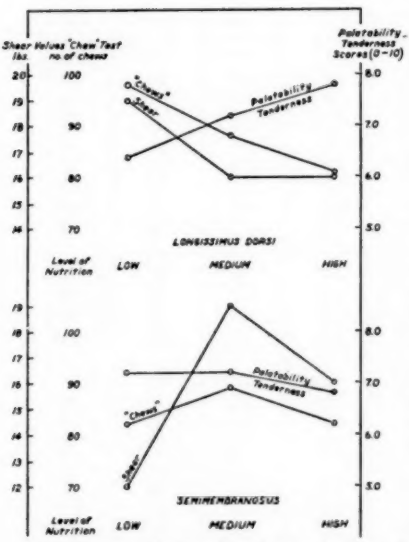


Figure 3. Mean values for tenderness of beef reared on three levels of nutrition as measured by palatability scores, the "chew" test, and shear force values on cooked meat.

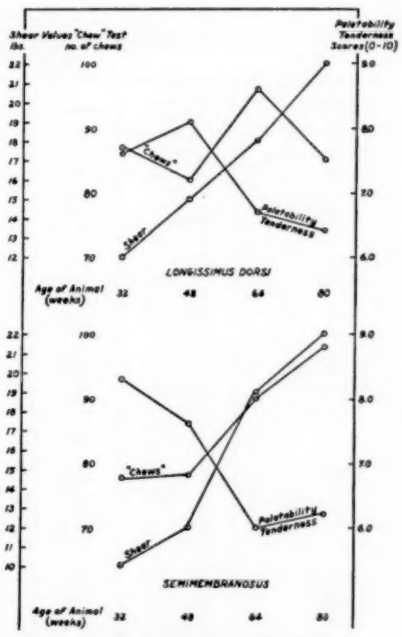


Figure 4. Mean values at four ages for tenderness by palatability scores, "chew" test, and shear force values on cooked beef.

ness than those on the other levels, no consistent or significant differences owing to the level of nutrition were observed in the other muscles (Table 6). *Age:* A consistent and highly significant decrease in juiciness of all 3 muscles with increase in age of the animals occurred (Table 7). It may have been related to the decrease in moisture with age.

Tenderness: Mean palatability scores for tenderness of individual roasts ranged from 3.7, *low average*, to 9.2, *excellent*. *Level of nutrition:* Significantly higher tenderness scores were recorded for *longissimus dorsi* roasts from the animals on the higher levels of nutrition than from those on the low level (Table 6). No differences in tenderness scores for the other muscles could be attributed to the level of feeding. *Age:* Tenderness scores of the *longissimus dorsi* muscles tended to decrease with age (Table 7). The decrease in tenderness scores of the *semimembranosus* muscle (the most-used muscle of those studied) with age was highly significant. *Muscles:* Mean score for the *psoas major* muscles was 7.7; those for the *longissimus dorsi* and *semimembranosus* were about equal, at 7.1.

"Chew" Values: The mean of the "chew" values of all of the roasts was 8.[1] (Table 6). *Level of nutrition:* The *longissimus dorsi* muscle tended to require fewer "chews" at the higher levels of feeding than at the low level, but the other muscles did not show this relationship (Table 6). *Age:* No mathematically significant differences in the "chew" values for the roasts could be attributed to the age of the animals although a trend toward decreased tenderness (increased number of "chews") with age from 48 to 80 weeks was noted for the *semimembranosus* muscle (Table 7). *Muscles:* The *psoas major* required the lowest number of "chews." The *longissimus dorsi* and *semimembranosus* were about equal in "chew" values.

TABLE 6

Effect of level of nutrition on mean palatability scores, number of "chews," and shear values of beef

Muscle	Level of Nutrition	Aroma	Flavor	Juiciness	Tenderness	No. of chews	Shear values
Longissimus dorsi[1]	Low	6.8	6.7	7.0	6.4	98	19
	Medium	6.6	7.3	7.4	7.2	88	16
	High	6.8	7.2	7.3	7.8	81	16
	Mean	6.7	7.1	7.2	7.1	89	17
Psoas major[2]	Low	6.7	6.9	6.8	7.9	64	9
	Medium	6.5	7.2	7.6	7.9	62	12
	High	6.6	7.3	7.2	7.9	58	10
	Mean	6.6	7.1	7.2	7.9	61	10
Semimembranosus[3]	Low	7.1	7.0	7.7	7.2	82	12
	Medium	6.9	7.5	7.8	7.2	89	19
	High	6.8	7.3	7.6	6.8	82	16
	Mean	6.9	7.3	7.7	7.1	84	16
Adductor[4]	Low	6.4	6.2	6.3	7.0	74	16
	Medium	6.5	7.0	7.1	6.8	85	20
	High	6.7	7.2	7.2	6.7	75	16
	Mean	6.5	6.8	6.9	6.8	80	17
Mean for level	Low	6.8	6.7	7.0	7.1	80	14
	Medium	6.6	7.3	7.5	7.3	81	17
	High	6.7	7.3	7.3	7.3	74	14

[1] Each value is the mean for 6-8 roasts by 6-7 judges.
[2] Each value is the mean for 3-5 roasts.
[3] Each value is the mean for 9-12 roasts.
[4] Each value is the mean for 4 roasts.

Shear values of cooked meat. *Shear:* Mean shear value of all of the cooked roasts was 14 pounds (Table 6). *Level of nutrition:* The differences in shear values for the *longissimus dorsi* and *psoas major* muscles after roasting could not be attributed to level of nutrition (Table 6). Shear values for the *semimembranosus* muscles from the animals on the medium level of feeding were significantly greater than from those on the low and high levels. *Age:* Shear force values increased consistently with the age of the animal (Table 7). The differences were highly significant for the *semimembranosus*, and significant for the closely adjoining *adductor* muscle. *Muscles:* As expected, the *psoas major* muscle had lower shear values, indicating greater tenderness, than did the *longissimus dorsi* or *semimembranosus* muscles. The values for the last two muscles were similar as they had been in the palatability and "chew" tests for tenderness.

Moisture of Cooked Meat: Mean moisture content of all of the cooked roasts was 67.3%. *Level of nutrition:* Moisture content in roasts from animals on the high level of feeding was 66.1%; in the roasts from animals on the medium level 67.7, and on the low level of feeding 68% (Table 8). The differences were not significant. Evidently cooking partially obliterated the differences found in the raw meat; namely, the moisture decreased significantly with increase in nutritive level. There were also no significant differences in juiciness scores owing to the level of feeding. *Age:* A consistent decrease in moisture with age occurred but it was not mathematically significant (Table 9). In the raw meat the differences were significant. The juiciness of the cooked meat decreased in the same manner as the moisture with age. *Muscle:* As in the uncooked meat, the *psoas major* muscle contained the least moisture after roasting, and the *longissimus dorsi* and *semimembranosus* contained nearly the same per cent.

TABLE 7

Effect of age of animal on mean palatability scores, number of "chews," and shear values of beef

Muscle	Age, weeks	Aroma	Flavor	Juiciness	Tenderness	No. of chews	Shear values
Longissimus dorsi[1]	32	6.2	7.5	7.8	7.6	87	12
	48	7.3	7.8	8.3	8.1	82	15
	64	6.4	6.7	6.8	6.7	96	18
	80	6.6	6.7	6.6	6.4	85	22
	Mean	6.6	7.2	7.4	7.2	88	17
Psoas major[2]	32	....	....	....	....	....	11
	48	6.8	8.0	8.0	8.6	62	8
	64	6.4	6.7	6.9	6.4	....	9
	80	6.6	6.7	6.6	8.0	60	16
	Mean	6.6	7.1	7.2	7.7	61	11
Semimembranosus[3]	32	7.2	7.8	8.3	8.3	78	10
	48	7.0	7.5	7.9	7.6	79	12
	64	7.0	7.0	7.1	6.0	90	19
	80	6.6	6.8	6.7	6.2	98	22
	Mean	7.0	7.3	7.5	7.0	86	16
Adductor[4]	32	....	....	....	....	....	....
	48	....	....	....	....	....	....
	64	6.5	6.9	7.1	7.3	74	13
	80	6.6	6.7	6.6	6.3	86	21
	Mean	6.6	6.8	6.8	6.8	80	17
Mean for age	32	6.8	7.6	8.1	8.0	82	11
	48	7.0	7.8	8.1	8.1	74	11
	64	6.6	6.8	6.9	6.6	87	15
	80	6.6	6.7	6.6	6.7	82	20
	Mean	6.8	7.3	7.5	7.4	81	14

[1] Each value is the mean for 4-8 roasts by 6-7 judges.
[2] Each value is the mean for 3-6 roasts.
[3] Each value is the mean for 7-10 roasts.
[4] Each value is the mean for 6 roasts.

Fat in Cooked Meat: The fat content of the cooked muscles ranged from 0.2 to 9.2% with a mean of 2.3%. *Level of nutrition:* A stepwise increase in fat content with increased levels of nutrition was observed (Table 8). The differences were highly significant for the *longissimus dorsi, semimembranosus* (and *adductor* muscles), and significant for the *psoas major.* In the meat from the animals on the low level of feeding the fat content was 1.2%, on the medium level 2.2%, and on the high level 3.5%. A similar difference in fat content was found in the raw meat. *Age:* An increase in fat content with age of the animal was highly significant in the *semimembranosus* muscle and the same trend was noted in the other 2 muscles (Table 9). *Muscle:* The *psoas major* muscle contained the highest percentage of fat, the *longissimus dorsi* the next, and the *semimembranosus* the least. The amount of fat in the cooked muscle followed closely the amount in the raw muscle.

RESULTS AND DISCUSSION

Effects of level of nutrition. From observations on 24 animals at 3 levels of nutrition it was found that levels of nutrition, equal to Morrison's Standards and higher, produced decided improvement in size and appearance of beef muscles from cattle of dairy breed. It is believed that the

TABLE 8

Effect of level of nutrition on mean moisture and fat content of cooked lean meat[1]

Level of nutrition	Moisture	Fat
	%	%
Low	68.0	1.2
Medium	67.7	2.2
High	66.1	3.5

[1] Each value is the mean of 20-32 roasts.

differences with level of feeding may be great enough to influence consumer acceptance in market sales.

In the raw, lean tissues from these animals the moisture was decreased and the fat content increased by better feeding. The decrease in moisture was not noted in the roasted meat, nor was it reflected in the palatability scores for juiciness. The higher fat content of the raw beef at the higher levels of nutrition carried over into the cooked meat. The total nitrogen content was unaltered by the level of nutrition. The effects of level of nutrition upon color, iron, and B_{12} content will be reported in papers which follow.

Differences in cooking losses owing to level of nutrition were negligible; total weight loss, drip, coagulum, evaporation losses were unchanged. Increased feeding did not lead to increased losses from the lean portion of the meat.

Regarding palatability, it will be noted that all of this dairy breed beef when roasted was rated average or higher on most characteristics. The flavor of the lean meat from the medium and high levels of feeding was scored higher than that from the low level. For one muscle, the *longissimus dorsi*, the tenderness scores were higher at the medium and high levels of nutrition than at the low level; this was verified by lower "chew" scores for the higher levels. Shear force values were variable and the evidence from them was inconclusive. But it is indicated that the flavor and tenderness of certain muscles of dairy beef may be improved by the method of feeding.

Effects of age of animal. An increase in size of the beef muscles with age was accompanied by a decrease in water content of the tissues; the fat content increased except at the low level of nutrition. Nitrogen, and some other nutrients, appeared to increase with the age of the animal.

TABLE 9

Effect of age of animal on mean moisture and fat content of cooked lean meat[1]

Age	Moisture	Fat
weeks	%	%
32	68.7	0.4
48	68.2	0.4
64	67.2	2.1
80	66.5	3.0
Mean	67.6	1.5

[1] Each value is the mean of 25-35 roasts.

Among the cooking losses, drip and coagulum losses decreased with age of the animal.

In the palatability scoring a preference was shown for the aroma and flavor of the lean beef at 48 weeks. With recognition that even a trained taste panel may vary in scoring between test periods, it appears that dairy beef reaches its highest palatability at about one year of age. Further tests of this type could provide further information as to optimum feeding periods and slaughter times.

Tenderness scores decreased with age, particularly for the *semimembranosus* muscle. The increased nitrogen content of the raw and increased shear force values for the cooked meat tend to support the finding of lowered tenderness with age; differences were not found, however, in the "chew" values with increased age.

If the demand is great enough and the cost of feeding feasible, it appears that high levels of nutrition can improve the size and appearance of beef from dairy herds. Some increase will result in the content of certain nutrients, without increase in cooking losses, and with some improvement in palatability. The effects of age of the animal need to be considered and observed when studies of the effects of nutrition are made.

SUMMARY

A study was made of the effects of three levels of nutrition and of age on the quality of beef from Holstein dairy cattle.

With increase in level of nutrition the weight of the muscles and fat increased in the raw meat, the moisture content decreased. There were no significant changes in total nitrogen content nor in shear force values.

With increase in age the carcass weight, fat, and nitrogen content increased in the raw meat, and the shear force values tended to increase.

The per cent weight, drip, coagulum, and evaporation losses during cooking of the hard-frozen roasts were not shown to be related to the nutrition of the animal.

The per cent weight and evaporation losses did not vary consistently with the age of the animal, but the per cent drip and coagulum tended to decrease with age.

In the cooked meat, as in the raw meat, the fat content increased with the level of nutrition. There were no consistent effects of level of nutrition on shear values, aroma, juiciness. Tenderness of the *longissimus dorsi* muscle may have been improved. The flavor of the beef from animals on higher levels of nutrition was preferred over that of beef from animals on the low level.

With increase in age, the fat content and shear values of the cooked meat increased as they had in the raw meat. There was also a small but consistent decrease in moisture. With increase in age, scores for aroma, flavor, juiciness, and tenderness tended to decrease after 48 weeks of age.

The palatability of roasts from dairy heifer beef were scored *acceptable* or higher by an experienced taste panel. Indications are that, if costs permit, the palatability of roasts from some muscles may be improved by high levels of nutrition, particularly in the younger animals.

Acknowledgments

The authors wish to express their appreciation to Dr. G. H. Wellington, Department of Animal Husbandry, who made studies on the whole body composition and carcass characteristics of the animals; from his dissections he furnished the stripped-out muscles used in this study. They are grateful also to the members of the palatability panel: Dr. W. L. Clark, Dr. H. D. Naumann, Dr. P. E. Ramstad, Dr. B. E. Sheffy, Dr. Betty F. Steele, Dr. J. J. Wanderstock, and Dr. G. H. Wellington.

LITERATURE CITED

1. Association of Official Agricultural Chemists, *Official Methods of Analysis.* 7th ed., Washington 4, D. C. 1950.
2. Brookes, A. J. Rearing dairy-bred calves for beef. *Agriculture* (formerly *J. Ministry Agr., England)*, **58**, 365-370 (1951).
3. Dunn, H. O. Further studies of the influence of three levels of nutrient intake on the growth and sexual development of young Holstein bulls. (Unpublished Ph.D. thesis, Cornell University, 1952.)
4. Jacobson, M., and Fenton, F. Effects of three levels of nutrition and age of animal on the quality of beef. II. Color, total iron content, and pH. *Food Research*, **21** (This issue).
5. Jacobson, M., and Fenton, F. Effects of three levels of nutrition and age of animal on the quality of beef. III. Vitamin B_{12} content. *Food Research*, **21** (This issue).
6. Lowe, Belle. Organoleptic tests developed for measuring the palatability of meat. *Proc. Sec. Annual Reciprocal Meat Conf.*, p. 111. National Live Stock and Meat Board. 1949.
7. Ma, T. S., and Zuzaga, G. Micro-Kjeldahl determination of nitrogen. *Ind. Eng. Chem., Anal. Ed.*, **14**, 280-282 (1942).
8. Musgrave, S. D. The influence of three levels of nutrient intake on the growth and sexual development of young Holstein bulls. (Unpublished Ph.D. thesis, Cornell University, 1951).
9. Snedecor, G. W., *Statistical Methods.* 4th ed., Iowa State College Press, Ames, Iowa. 1948.
10. Sorensen, A. M. The growth and sexual development of young Holstein heifers as influenced by three levels of nutrition. (Unpublished Ph.D. thesis, Cornell University, 1953.)
11. Wellington, G. H., Reid, J. T., Bratzler, L. J., and Miller, J. I. The use of antipyrene in nutrition and meat studies with cattle. *J. Animal Sci.*, **15**, 76-85 (1956).

EFFECTS OF THREE LEVELS OF NUTRITION AND AGE OF ANIMAL ON THE QUALITY OF BEEF. II. COLOR, TOTAL IRON CONTENT, AND pH

MARION JACOBSON* AND FAITH FENTON
State College of Home Economics and State Agricultural Experiment Station, Cornell University, Ithaca, New York

(Manuscript received September 8, 1955)

Although the redness of the color of raw beef has been recognized as an important quality factor, its control and management are incompletely understood. Published studies indicate conflicting evidence as to the effects of energy and mineral intake upon the color of lean meat.

The purpose of this study was to determine the color, total iron content, and pH of beef from Holstein heifers which had been reared under controlled feeding throughout their growth. Three stripped-out muscles *(longissimus dorsi, psoas major,* and *semimembranosus)* were studied. The animals had been fed on one of three levels of total digestible nutrients and minerals from the age of one week until slaughtered at 32, 48, 64, or 80 weeks of age *(1)*.

Previous investigations. In a study of the effects of diets with and without liver upon the muscle hemoglobin (myoglobin) levels of dogs, Whipple *et al. (9)* found that the myoglobin content was affected by dietary factors but that it was less labile than was the hemoglobin content of the blood.

Grass, or grass plus grain, increased the myoglobin content of beef above that in meat from animals fed only grain, according to Shenk *et al. (4)*. These workers, however, considered that the differences observed might have been owing to the greater activity of the grazing animals during foraging rather than to the feed.

Measurements of the color values of beef from two-year-old steers by Longwell *(3)* indicated no effect of grass feeding upon the color of the lean meat. The animals had been fed on grass or grain for 140 days prior to slaughter.

Variation in the plane of nutrition of hogs did not induce observable changes in the myoglobin of pork, according to Lawrie *(2)*. The effects of the ages of the animals may have influenced these observations. Since the hogs were reared to a constant slaughter weight, the animals on the lower planes of feeding were older when analyzed than those fed on the higher planes.

History of meat. This report is the second on a section of a larger study. Part I dealt with the moisture, fat, and nitrogen contents as well as palatability and cooking data *(1)*. The study was a part of an investigation of the effects of 3 levels of nutrition upon the development of Holstein heifers carried out by the Department of Animal Husbandry, Cornell

*This is part of a study which was submitted in partial fulfillment of the requirements for the Ph.D. degree by Marion Jacobson. Present address: School of Home Economics, Purdue University, Lafayette, Indiana.

TABLE 1

Daily mineral[1] intakes of Holstein heifers on three levels of nutrition at various ages[2]

Age	Level of nutrition	Iron	Copper	Cobalt
weeks		*mg.*	*mg.*	*mg.*
4	Low	70	11	0.3
	Medium	60	11	0.2
	High	130	12	0.5
16	Low	290	44	2.3
	Medium	370	55	4.5
	High	1020	113	10.4
32	Low	410	64	3.6
	Medium	670	112	5.9
	High	1700	203	18.5
48	Low	560	89	3.6
	Medium	830	143	6.0
	High	1860	228	22.2

[1] Each mineral was given in the form of a hydrated sulfate salt.
[2] Adapted from Sorensen, A. M., Jr., The Growth and Sexual Development of Young Holstein Heifers as Influenced by Three Levels of Nutrition. (Unpublished thesis, Cornell University, 1953.)

University *(6, 8)*. The intakes of minerals are recorded in Table 1; other aspects of the feeding and management of the animals were described by Sorensen *(6)*.

The 24 carcasses studied included those from 2 animals on each level of feeding at each slaughtered age (32, 48, 64, and 80 weeks). The objective color, total iron content, and pH values of raw samples of the three stripped-out muscles were obtained as well as objective color readings on the meat after it had been roasted. Roasts of uniform weight and dimensions were cooked from the hard-frozen state (−12° C., 10° F.) in a 149° C. (300° F.) test oven to an internal temperature of 69° C. (156° F.). After removal from the oven each roast was allowed to cool to 60° C. (140° F.) prior to sampling. The history of the meat and the treatment of the samples have been described in Part I *(1)*.

EXPERIMENTAL

Determination of color. *Raw Beef:* From the stripped-out muscles *(longissimus dorsi, psoas major,* and *semimembranosus)* which had been trimmed of exterior fat and membrane, uniformly placed samples were removed as described in Part I *(1)*. The slice used for the measurement of the color of the raw meat was cut one inch in thickness and at right angles to the direction of the muscle fibers. The samples were wrapped and held in frozen storage until the roasts from the same muscle were cooked.

Just prior to cooking of the roasts, the raw meat from which slices for color tests was to be cut was thawed at room temperature without removal from freezer wrappings. While the meat was cold, a slice was cut so as to expose fresh surfaces at least one-half inch below the previous surfaces of the meat.

The color of the slices was read on a Hunter Color and Color-Difference Meter after 30 minutes' exposure to the air. The time of exposure was carefully controlled in order to obtain a comparable degree of formation of oxymyoglobin.

The color plate used in standardizing the color meter for the reading of the color of the raw meat had the following values: Rd 24, a ± 18.2 and b ± 8.0. Results are reported as hue (a/b ratio).

Cooked Beef: A one-half-inch slice was cut at right angles to the fibers from the center of each cooked roast. The freshly-cut slices of meat were exposed to the air for 30 minutes prior to the color readings.

The plate used in adjusting the color difference meter for reading the color of the cooked samples had the following values: Rd 2.0, a ± 3.8, and b ± 6.5.

Determination of total iron content. Iron determinations were made on the uncooked meat only. The samples were held in frozen storage until analyzed.

Preparation of Slurries for Iron Determinations: The surfaces of the meat were cut away and discarded; then small cubes were sliced from the inner portion for samples. New stainless steel knives were used for cutting to avoid possible contamination with iron. Approximately 50 g. of the cubes were weighed into 400-ml. beakers. The meat was covered with glass-distilled water, and a glass triangle and watch glass were placed over each beaker. Samples were simmered for 30 minutes. While the mixture was still warm, it was transferred to a blender cup and blended for 2 minutes. A new cup and blade assembly was used to avoid possible contamination with iron from this source. The blended mixture was transferred to a 2-liter Erlenmeyer flask, using hot glass-distilled water for the transfer. Thirty ml. of concentrated hydrochloric acid were added. Each flask was covered with a beaker and heated on a steam bath for 3 hours, with frequent agitation of the contents by swirling. When the cooked slurry had been cooled to room temperature, it was diluted to 500 ml. and transferred to a one-liter Erlenmeyer flask, sealed with wax film, and stored at room temperature. Assay of iron in slurries: The procedure was an adaptation of the thiocyanate method of Stugart *(7)*.

A 25-ml. sample was pipetted from the well-mixed slurry into a 300-ml. Kjeldahl flask for the wet-ashing procedure. If fat had collected at the top of the slurry, the slurry was heated to 42° C. and the measurement made with a pipette calibrated for that temperature. After addition of pyrex beads, the sample was heated with 3 ml. of concentrated sulfuric acid and additions of nitric acid until it was clear. Superheating was avoided. Nitrosyl-sulfuric acid was removed by additions of glass-distilled water followed by reheating. The digested sample was diluted to 50 ml.

Five or 10 ml. of sample were introduced to a separatory funnel and the volume brought to 20 ml. with glass-distilled water. One drop of concentrated nitric acid and one drop of potassium permanganate were added. Fifteen ml. of isoamyl alcohol and 5 ml. of 20% potassium thiocyanate were added and the mixture was shaken 30 seconds, during the development of the color.

The percentage transmittance of the developed red color was obtained within 5 minutes by use of an Evelyn colorimeter (490 mμ filter). Isoamyl alcohol was used as the reference solution. The readings were interpreted as iron content by reference to a standard curve and by calculation for the dilution of the sample. All samples were tested in duplicate.

Iron content was calculated on both the moist and the dry, fat-free basis. Moisture and fat contents have been reported in a previous paper from this laboratory *(1)*.

Determination of pH. Determinations of pH were made on the raw beef by use of a Beckman pH Meter, model G, which had been equipped with durable and shielded external electrodes.

In preliminary trials the readings of pH were made on samples of the meat in each of 3 forms—solid meat, ground meat, and ground meat to which an equal weight of water had been added. According to the first method the glass electrodes were pressed firmly but gently against the surface of a freshly-cut slice of solid meat; in the second method electrodes were inserted to a distance of ½-inch into a sample of twice-ground raw meat; and in the third method 10 g. of ground meat were mixed well with 10 ml. of distilled water prior to making the reading. The 3 methods yielded almost identical readings for samples from the same piece of meat. The use of a solid sample, however, yielded the least consistent results. For accuracy and convenience, the determinations reported in this study were made on undiluted ground meat.

Statistical methods. The statistical design and treatment of data were the same as reported in Part I *(1)*.

RESULTS AND DISCUSSION

Raw meat. A summary of the mathematical significance of the data is given in Table 2.

TABLE 2

Summary of mathematical significance[1] of the data

Factor studied	Level of nutrition			Age of animal		
	Longissimus dorsi	*Psoas Major*	*Semimembranosus*	*Longissimus dorsi*	*Psoas Major*	*Semimembranosus*
Raw meat						
Hue	0	0	[3]	0	[3]	[3]
Iron content	0	[2]	[3]	[3]	[3]	[3]
pH	0	0	0	0	0	0
Cooked meat						
Hue	0	0	[2]	0	0	[3]

[1] 0—not significant.
[2] —significant, 5% level.
[3] —highly significant, 1% level.

Color of raw meat. The color of the meat is reported as hue, or a/b ratio. The mean hue values of all of the uncooked roasts was 1.72.

The hue values for the uncooked *longissimus dorsi* and *psoas major* muscles were not statistically significantly different owing to the level of nutrition (Table 3). In the *semimembranosus* muscles, however, the differences in hue values were highly significant (1% level).

Level of Nutrition: That the redness of the color of the raw meat increased with increases in level of nutrition of the animal was noted consistently in subjective observations. Objective confirmation of the relation-

TABLE 3

Effect of level of nutrition on mean values for hue, iron content, and pH of raw beef from Holstein heifers

Muscle	Level of nutrition	Hue[1] (a/b ratio)	Iron[2]		pH[3]
			Moist basis	Dry, fat-free basis	
			mg./100 g.		
Longissimus dorsi	Low	1.70	1.22	5.13	5.66
	Medium	1.75	1.38	5.67	5.60
	High	1.88	1.56	6.46	5.61
	Mean	1.78	1.39	5.75	5.62
Psoas major	Low	1.68	1.40	6.19	5.62
	Medium	1.74	1.65	7.10	5.53
	High	1.84	1.87	8.02	5.62
	Mean	1.75	1.64	7.10	5.59
Semimembranosus	Low	1.48	1.24	5.22	5.59
	Medium	1.67	1.60	6.44	5.58
	High	1.77	1.92	7.76	5.52
	Mean	1.64	1.59	6.47	5.56
Mean for level	Low	1.62	1.29	5.51	5.62
	Medium	1.72	1.54	6.40	5.57
	High	1.83	1.78	7.41	5.58

[1] Each value is the mean of hue readings on 5-7 muscles from as many different animals.
[2] Each value represents the mean of duplicate determinations of iron on 7 or 8 muscles from as many animals.
[3] Each value represents the mean of the pH values of 6-8 muscles from as many animals.

ship was probably limited by intramuscular deposits of fat, as well as by unavoidable irregularities in sampling of the *longissimus dorsi* and *psoas major* muscles. It was sometimes necessary to patch the samples, particularly from the relatively small *psoas major* muscles. The *semimembranosus* muscles were large enough and relatively uniform throughout so that it was possible to obtain samples that were in turn relatively uniform in size and structure. It is possible also that the muscle which has the most activity (of these, the *semimembranosus)* is most easily affected by such factors as level of nutrition.

Age of Animal: The relationship of redness of the beef to the age of the animal appeared to vary from muscle to muscle (Table 4). In the

TABLE 4

Effect of age of animal on mean values for hue, iron content, and pH of raw beef from Holstein heifers

Muscle	Age, weeks	Hue[1] (a/b ratio)	Iron[2]		pH[3]
			Moist basis	Dry, fat-free basis	
			mg./100 g.		
Longissimus dorsi	32	1.41	0.94	3.91	5.75
	48	1.82	1.27	5.15	5.60
	64	1.70	1.46	6.08	5.55
	80	2.04	1.66	6.94	5.56
	Mean	1.74	1.33	5.52	5.62
Psoas major	32	1.86	1.24	5.47	5.70
	48	1.47	1.60	6.75	5.58
	64	1.50	1.90	8.23	5.49
	80	2.00	1.81	7.71	.....
	Mean	1.71	1.64	7.04	5.59
Semimembranosus	32	1.32	1.14	4.65	5.58
	48	1.54	1.38	5.56	5.67
	64	1.69	1.76	7.25	5.50
	80	1.84	2.08	8.46	5.49
	Mean	1.60	1.59	6.48	5.56
Mean for age	32	1.53	1.11	4.67	5.68
	48	1.61	1.42	5.82	5.62
	64	1.63	1.71	7.19	5.51
	80	1.96	1.83	7.70	5.52

[1] Each value is the mean of hue readings on 3-6 muscles from as many different animals.
[2] Each value is the mean of duplicate determinations of iron on 6 muscles from different animals, except, at 32 weeks, 3 *longissimus dorsi* muscles were analyzed.
[3] Each value for pH represents measurements made on 3 to 6 muscles from different animals.

longissimus dorsi muscles the a/b values tended to increase with age; the differences, however, were not statistically significant. The a/b values for the *psoas major* muscles did not increase with age. In the *semimembranosus* muscles the increase in hue values with increased age was highly significant. Again, the most exercised muscle was affected the most by age as it had been by level of nutrition.

pH values. The range of pH readings on the 63 uncooked muscles ranged from 5.44 to 6.00, with almost all of the values between 5.45 and 5.70 (Tables 2 and 3). The mean pH value was 5.59. No relationship was

observed between the small variations in pH and level of nutrition, age of the animals, or type of muscle. Since the meat had been aged 7 days prior to frozen storage, the normal post-slaughter decrease in pH had probably occurred before the tests were made. The variations in hue values of the raw meat were not explained by variations in the pH of the muscles.

Iron content. The mean iron content of all of the muscles at all of the ages was 6.48 mg. per 100 g. with a range of from 3.40 to 11.87 mg. per 100 g. on the dry, fat-free basis.

Level of Nutrition: The iron content of the muscles was increased by the higher levels of nutrition (Table 3). In the *longissimus dorsi* muscles a stepwise increase was observed, but the differences were not statistically significant. In the *psoas major* muscles differences were significant (5% level) and in the *semimembranosus* muscles the increases were highly significant.

The beef from animals which had been fed on the highest plane of total digestible nutrients and minerals contained 20 to 30% more iron than did that from animals fed at the lowest level. The beef from animals on the medium level of nutrition had intermediate iron content.

Age of Animal: The iron content of the muscles increased with the age of the animal (Table 4). The differences in values owing to age were highly significant for the *longissimus dorsi*, significant for the *psoas major*, and highly significant for the *semimembranosus* muscles. These differences were visible subjectively as increases in redness of color.

Iron Content and Color: Increases in the hue values of the raw beef were usually accompanied by increases in the content of iron in the tissues

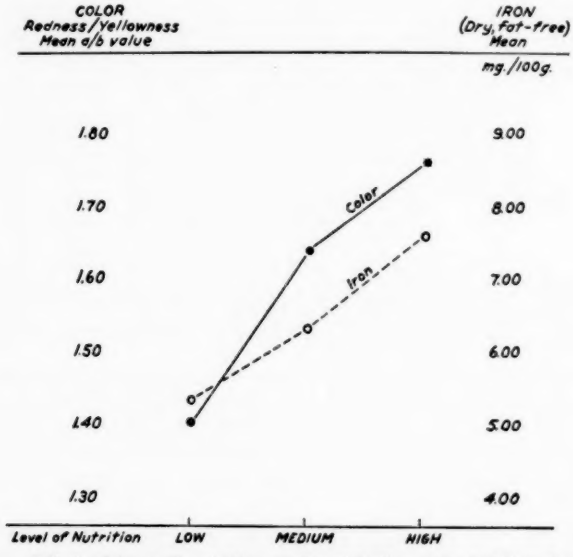


Figure 1. Effect of level of nutrition on the objective color (a/b) and iron content of raw beef from the semimembranosus muscles of heifers.

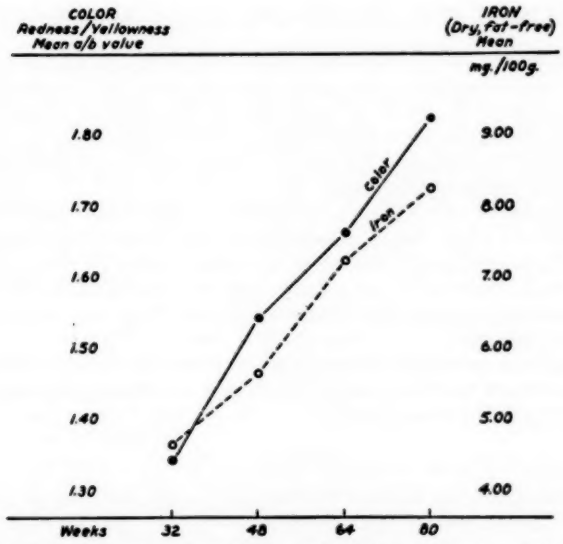


Figure 2. Effect of age on the objective color (a/b) and iron content of raw beef from the semimembranosus muscles of heifers.

(Tables 3 and 4). The relationships in the *semimembranosus* muscles between the hue values (a/b ratio) and the iron content at the 3 levels of nutrition is given in Figure 1. The relationship of hue to iron content at 4 ages is shown in Figure 2.

TABLE 5

Effect of level of nutrition on mean values for hue of cooked beef from Holstein heifers

Muscle	Level of nutrition	Hue[1] (a/b ratio)
Longissimus dorsi	Low	0.81
	Medium	0.78
	High	0.88
	Mean	0.82
Psoas major	Low	1.04
	Medium	1.06
	High	1.20
	Mean	1.10
Semimembranosus	Low	0.84
	Medium	0.86
	High	1.04
	Mean	0.91
Mean for level	Low	0.90
	Medium	0.90
	High	1.04

[1] Each value is the mean of hue readings on 3-8 roasts from different animals.

Color of cooked meat. The mean hue values of all of the cooked meat was 0.94.

Level of Nutrition: Analysis of the hue values of the cooked roasts from the *longissimus dorsi* and *psoas major* muscles indicated no significant differences owing to the level of nutrition of the animals (Table 3). The roasts from the *semimembranosus* muscles of the animals on the high levels of feeding had significantly higher a/b values than those from animals on the two lower levels of nutrition.

Age of Animal: The hue values of the cooked roasts from the *longissimus dorsi* and *psoas major* muscles were not significantly changed with increase of age of animals from 32 to 80 weeks (Table 4). The cooked meat from the *semimembranosus* muscles of the 80-week animals, however, had highly significant higher a/b values than that from the same muscle in the younger animals.

Although the most intensely red raw meat was observed to yield roasted meat of a deeper red hue, small differences in redness apparently were obliterated by the cooking process.

SUMMARY

Measurements were made of the objective color, pH, and total iron content of 3 muscles *(longissimus dorsi, psoas major,* and *semimembranosus)* from 24 Holstein heifers as affected by 3 levels of nutrition (varying primarily in total digestible nutrients and minerals) and age of the animals.

An increase in redness or hue (Hunter a/b ratio) owing to level of nutrition was significant for the *semimembranosus* muscle both before and

TABLE 6

Effect of age of animal on mean values for hue of cooked beef from Holstein heifers

Muscle	Age, weeks	Hue[1] (a/b ratio)
Longissimus dorsi	32	0.79
	48	0.78
	64	0.80
	80	0.92
	Mean	0.82
Psoas major	32	.....
	48	1.17
	64	1.10
	80	1.08
	Mean	1.12
Semimembranosus	32	0.80
	48	0.80
	64	0.86
	80	1.19
	Mean	0.91
Mean for age	32	.....
	48	0.92
	64	0.92
	80	1.06

[1] Each value is the mean of hue readings on roasts from 3-6 muscles from different animals.

after cooking. Similar trends observed subjectively in the *longissimus dorsi* and *psoas major* muscles were not verified by objective color measurements on the raw or the cooked meat.

A highly significant increase in the hue of both the raw and roasted beef from the *semimembranosus* muscles was noted with the increase of age of the animals from 32 to 80 weeks. This finding was also observed subjectively. The objective color values of the *longissimus dorsi* and *psoas major* muscles did not increase significantly with the age of the animals.

Color variations could not be explained on the basis of differences in pH. Little variation in the pH of the muscle from these carcasses which had been aged for 7 days was observed.

The total iron content of the muscles increased with the increase in level of nutrition. The increase was observed as a trend in the values for the *longissimus dorsi*. The differences were significant for the *psoas major* and highly significant for the *semimembranosus* muscles. Beef from animals fed at the high level contained 20 to 30% more iron than that produced on the low level of feeding.

The iron content of the muscles also increased with the age of the animals from 32 to 80 weeks. The differences in iron content owing to age were highly significant for the *longissimus dorsi* and *semimembranosus* muscles, and significant for the *psoas major* muscles.

The redness and iron content of raw beef were increased by both level of nutrition and age of the animal, particularly in the *semimembranosus* muscle.

Acknowledgments

Supplementary funds for the iron determinations were made available through the School of Nutrition, Cornell University, Dr. Leonard A. Maynard, Director.

The authors also wish to thank Dr. Frances A. Johnston for advice on the method of analysis for iron and for the use of specialized laboratory facilities for the analyses.

LITERATURE CITED

1. Jacobson, M., and Fenton, F. Effects of three levels of nutrition and age of animals on the quality of beef. I. Palatability, cooking data, moisture, fat, and nitrogen. *Food Research*, 21 (This issue).
2. Lawrie, R. A. Some observations on factors affecting myoglobin concentrations in muscle. *J. Agri. Sci.*, 40, 356-366 (1950).
3. Longwell, J. H. Color of lean of beef as affected by grass and grain feeding. W. Va. Agr. Expt. Sta., *Bull. 274* (1936).
4. Shenk, J. H., Hall, J. L., and King, H. H. Spectrophotometric characteristics of hemoglobins. I. Beef blood and muscle hemoglobins. *J. Biol. Chem.*, 105 741-752 (1934).
5. Snedecor, G. W., *Statistical Methods.* 4th ed., Iowa State College Press, Ames, Iowa. 1948.
6. Sorensen, A. M. The growth and sexual development of young Holstein heifers as influenced by three levels of nutrition. (Unpublished Ph.D. thesis, Cornell University, 1953.)
7. Stugart, R. Determination of iron in milk and other biological materials. *Ind. Eng. Chem., Anal. Ed.*, 3, 390-393 (1931).
8. Wellington, G. H. Body composition and carcass changes of young Holstein cattle. (Unpublished Ph.D. thesis, Cornell University, 1954.)
9. Whipple, G. H., Groth, A. H., and Robscheit-Robbins, F. S. Muscle hemoglobin concentration during growth as influenced by diet factors. *Am. J. Physiol.*, 87, 185-191 (1928).

EFFECT OF THREE LEVELS OF NUTRITION AND AGE OF ANIMAL ON THE QUALITY OF BEEF. III. VITAMIN B_{12} CONTENT

MARION JACOBSON[a] AND FAITH FENTON
New York State College of Home Economics and State Experiment Station, Cornell University, Ithaca, New York

(Manuscript received September 8, 1955)

The study reported herein completes the report of observations made on the quality and nutritive value of beef from Holstein heifers reared on 3 levels of nutrition to several ages. The first report was on shear values, moisture, fat and nitrogen content, cooking losses and palatability *(1)*; the second was on color (hue), total iron content, and pH *(2)*.

The purpose of this part of the study was to determine the effect of the level of nutrition and age on the vitamin B_{12} content of the *longissimus dorsi* and *semimembranosus* muscles from animals slaughtered at 32, 48, and 64 weeks of age. The feeding and management of the animals and handling and sampling of the meat are given in Part I of this study *(1)*.

Studies found in the literature on the storage of vitamin B_{12} in mammalian tissues had been made on rats. Extensive storage of the vitamin in the liver was reported by Scheid *(3)*. After administration of radioactively tagged B_{12}, prolonged storage of the vitamin in the pancreas and concentration in the rat's kidney was observed by Harte *(2)* and small amounts of radioactivity were found in the muscle.

Because of the extensive synthesis of the vitamin in the rumen of cattle, the storage in the tissue of these animals is of interest. No report has been found in the literature of the effects of energy and mineral intake or age upon the vitamin B_{12} storage in the lean muscle tissues of large meat-producing animals.

VITAMIN B_{12} ANALYSIS

Preparation and dilution of samples. Duplicate 5-g. samples were cut from each muscle to be assayed. Each sample was rinsed into a blender cup with 75 ml. of a phosphate buffer solution (pH 6.7-6.8). The meat and buffer mixture was homogenized for 5 minutes, after which the sample was transferred to a 400-ml. Erlenmeyer flask. Two mg. of sodium cyanide were added to each flask. Preliminary work had indicated that this amount of sodium cyanide was neded to change the vitamin from its inactive forms. Each mixture was swirled, stoppered, autoclaved for 30 minutes at 15 pounds pressure, and cooled to room temperature. The autoclaved, cooled sample was diluted to 200 ml., mixed with ½-teaspoon of filter aid, and filtered into an Erlenmeyer flask.

Aliquots of the filtered solution were further diluted so that 1 ml. of the extract contained 1 mg. of meat. The use of 1 to 5 ml. of this final dilution per assay tube resulted in densities of growth of the organism which were within measurable limits. These amounts were transferred in duplicate to the assay tubes.

Standard curve. Solutions containing 0.005, 0.02, and 0.05 mcg. of B_{12} per ml. were prepared daily from refrigerated stock solutions of the vitamin. Duplicate tubes con-

[a] Part of a study made in partial fulfillment of the requirements for the Ph.D. degree. Present address: School of Home Economics, Purdue University, Lafayette, Indiana.

taining 0.005, 0.010, 0.015, 0.020, 0.025, 0.040, 0.050, 0.060, 0.080, 0.100, 0.150, 0.200, and 0.250 meg. of B_{12} were pipetted for use in plotting the standard curve. Four tubes were left as blanks.

Microbiological assay. The microbiological method used for assay of vitamin B_{12} in the beef was a modification of the procedure of Peeler *(5)*.

Inoculum: Lactobacillus leichmanii 4797 (A. T. C. C.) which was used as a culture, was maintained in a stab medium made up of the basal medium with added liver extract, agar, and water. Every 2 weeks the culture was transferred to a broth medium enotaining Bactotryptone, liver extract, glucose, and K_2HPO_4. After 24 hours it was returned to the stab medium. In preparation of the inoculum, the organism was grown for 24 hours in a broth of the same composition as the stab medium, with the omission of the agar. After centrifuging, the inoculum was resuspended by dilution in sterile physiological saline solution to a density which read "60" on the galvanometer scale of a Coleman spectrophotometer and at a wave length of 650 mμ.

Basal Medium: The medium employed, which was a modification of that used by Daniel *et al. (1)*, contained filtered and decolorized tomato juice, amino acids, salts, vitamins other than B_{12}, glucose, and polyoxyethylene sorbitan monoleate. After the constituents had been combined, the pH was adjusted to 5.5, by additions of 20% potassium hydroxide in small amounts with constant mixing. The medium was made up to volume with distilled water, steamed for 5 minutes, and cooled to room temperature.

Inoculation and Incubation: The extracted samples of vitamin B_{12} solutions in the assay tubes were brought to a volume of 5 ml. with distilled water. Five ml. of the basal medium were dispensed into each tube. The tubes were covered with glass caps, steamed for 15 minutes, and cooled to room temperature before inoculation. Each tube was inoculated aseptically with a loopful of well-mixed inoculum.

The assay was incubated at 37° C. for 16 to 18 hours, until the tube containing 0.100 meg. of vitamin B_{12} read "30" on the scale of the spectrophotometer.

Calculation of the Vitamin Content: The turbidity of the contents of the assay tubes was read against that of the inoculated blank by use of a Coleman spectrophotometer which had been adjusted to a wave length of 650 mμ. Calculation of the vitamin content was made by reference to the standard curve and multiplication by the factor for dilution. The vitamin values (in μg. per 100 g. of meat) were also calculated on the dry, fat-free basis. The moisture and fat contents of these muscles has been reported previously *(1)*.

After the filtrates of the samples were adjusted to pH 12 and steamed for 30 minutes to destroy the vitamin, subsequent assays showed no growth owing to the stimulation of the organism by desoxyribosides.

Statistical methods. The statistical design and treatment of data were the same as reported in Part I *(1)*.

RESULTS AND DISCUSSION

Content of B_{12}. The mean value of the vitamin B_{12} content on the moist basis for all of the muscles was 1.40 μg. per 100 g. and 5.77 μg. per 100 g. on the dry, fat-free basis. The individual values obtained in duplicate for the *longissimus dorsi* and *semimembranosus* muscles from the Holstein heifers reared on the 3 levels of nutrition and to 3 ages were recorded in Table 1. There was good agreement between values obtained for two samples from the same muscle.

Level of Nutrition: Beef from the animals which had been reared on the 2 higher levels of nutrition contained more vitamin B_{12} than did that from animals on the low level of feeding (Table 2). This trend was not statistically significant for the *longissimus dorsi* muscles; however, it was significant (5% level) for the *semimembranosus* muscles. The difference in values owing to feeding was greater at the younger ages, approaching equality by the time the animals were 64 weeks of age (Table 1).

TABLE 1

Vitamin B_{12} content[1] of beef reared on three levels of nutrition to ages of 32, 48, or 64 weeks

Muscle	Level of Nutrition	Animal	B_{12} at 32 weeks		Animal	B_{12} at 48 weeks			B_{12} at 64 weeks	
			Moist basis	Dry, fat-free basis		Moist basis	Dry, fat-free basis		Moist basis	Dry, fat-free basis
			μg./100 g.			*μg./100 g.*			*μg./100 g.*	
Longissimus dorsi	Low	7	0.74	3.38	13	0.70	2.88	19	1.69	6.98
	Low	8	0.98	4.39	14	1.08	4.56	20	1.54	6.45
	Medium	9	1.46	5.92	15	0.89	3.56	21	2.04	8.44
	Medium	10	1.16	4.48	16	1.74	7.10	22	1.52	6.26
	High	11	1.56	6.30	17	0.98	3.95	23	1.48	6.23
	High	12	1.53	6.32	18	1.80	7.08	24	1.78	7.34
Semimembranosus	Low	7	0.82	3.55	13	1.07	4.30	19	1.22	5.98
	Low	8	1.02	4.20	14	0.89	3.68	20	1.46	6.26
	Medium	9	1.34	5.38	15	1.68	6.60	21	2.26	9.32
	Medium	10	1.60	6.50	16	1.34	5.45	22	1.48	6.16
	High	11	1.52	6.16	17	1.10	4.36	23[2]	1.44	5.98
	High	12	1.75	7.06	18	1.82	7.30	24	1.97	7.91

[1] Each value is the mean of duplicate determinations made on each muscle.
[2] Animal 23 grew less well than other animals fed at the high level.

TABLE 2

Effect of level of nutrition on the mean content of vitamin B_{12} in two muscles from Holstein heifers

Muscle	Level of nutrition	Vitamin B_{12} content		Vitamin B_{12} content	
		Moist basis		Dry, fat-free basis	
		Range	Mean	Range	Mean
		μg./100 g.		*μg./100 g.*	
Longissimus dorsi	Low	0.70-1.69	1.12	2.88-6.98	4.72
	Medium	0.89-2.04	1.47	3.56-8.44	6.27
	High	0.98-1.80	1.52	3.95-7.34	6.33
Semimembranosus	Low	0.82-1.46	1.08	3.55-6.26	4.66[1]
	Medium	1.34-2.26	1.62	5.38-9.32	6.58
	High	1.10-1.97	1.60	4.36-7.91	6.46

[1] Vitamin B_{12} content of *semimembranosus* varied significantly (5% level) with level of nutrition.

Age of Animals: The increase in vitamin B_{12} content at 64 weeks over that at 32 and 48 weeks was significant in both the *longissimus dorsi* and *semimembranosus* muscles (Table 3).

SUMMARY

Measurement was made of the vitamin B_{12} content of the *longissimus dorsi* and *semimembranosus* muscles from 18 Holstein heifers which had been reared on one of three levels of nutrition.

At the ages of 32 and 48 weeks the muscle tissue from animals reared on the low level of feeding contained significantly less B_{12} than did the muscle from the animals reared on the medium or high levels of feeding. The values for the muscles from the animals on the three levels of nutrition approached equality by the time the animals were 64 weeks of age.

Both the *longissimus dorsi* and the *semimembranosus* muscles from animals slaughtered at 64 weeks of age contained significantly more vitamin B_{12} than did these muscles from animals slaughtered at 32 or at 48 weeks of age.

Acknowledgment

The authors wish to express their appreciation to Dr. L. C. Norris, Professor of Poultry Nutrition, Cornell University, for his generous advice and for the use of his laboratory equipment in making the vitamin B_{12} analyses.

TABLE 3

Effect of age of animal on the mean vitamin B_{12} in two muscles from Holstein heifers

Muscle	Age of Animal	Vitamin B_{12} content		Vitamin B_{12} content	
		Moist basis		Dry, fat-free basis	
		Range	Mean	Range	Mean
		μg./100 g.		*μg./100 g.*	
Longissimus dorsi	32	0.74-1.56	1.24	3.38-6.32	5.13[1]
	48	0.70-1.80	1.20	2.88-7.10	4.86
	64	1.48-2.04	1.67	6.23-8.44	6.95
Semimembranosus	32	0.82-1.75	1.34	3.55-7.06	5.48[1]
	48	0.89-1.82	1.32	3.68-7.30	5.28
	64	1.22-2.26	1.64	5.98-9.32	6.93

[1] Vitamin B_{12} content of both muscles varied significantly (5% level) with age.

LITERATURE CITED

1. Daniel, L. J., Scott, M. L., Heuser, G. F., and Norris, L. C. Further studies of unidentified factors required in the nutrition of *Lactobacillus casei*. *J. Biol. Chem.*, **174**, 71-79 (1948).
2. Harte, R. A., Chow, B. F., and Barrows, L. Storage and elimination of vitamin B_{12} in the rat. *J. Nutr.*, **49**, 669-678 (1953).
3. Jacobson, M., and Fenton, F. Effects of three levels of nutrition and age of animal on the quality of beef. I. Palatability, cooking data, moisture, fat, and nitrogen. *Food Research*, **21** (This issue).
4. Jacobson, M., and Fenton, F. Effects of three levels of nutrition and age of animal on the quality of beef. II. Color, total iron content, and pH. *Food Research*, **21** (This issue).
5. Peeler, H. J., Yacowitz, H., and Norris, L. C. The microbiological assay for vitamin B_{12} using *Lactobacillus leichmanii*. *Proc. Soc. Expt. Biol. and Med.*, **72**, 515-521 (1949).
6. Scheid, H. E., Andrews, M. M., and Schweigert, B. S. Liver storage of vitamin B_{12} by the rat. *Proc. Soc. Expt. Biol. and Med.*, **78**, 558-560 (1951).
7. Snedecor, G. W., *Statistical Methods*. 4th ed., 1948, Iowa State College Press, Ames, Iowa.
8. Sorensen, A. M. The growth and sexual development of young Holstein heifers as influenced by three levels of nutrition. (Unpublished Ph.D. thesis, Cornell University, 1953.)

INVESTIGATION OF COPPER COMPLEXES CAUSING CLOUDINESS IN WINES. I. CHEMICAL COMPOSITION

C. E. KEAN* AND G. L. MARSH
Department of Food Technology, University of California, Davis, California

(Manuscript received December 19, 1955)

Formation of undesirable turbidities and sediments due to the presence of small amounts of metals in white wines has been, and still is, a major problem of the wine industry. The present study is concerned with the chemical composition of the actual copper-cloud material, and the factors which affect copper-cloud formation and composition.

Most early wineries had trouble with cloudy wines due to the unavoidable presence of iron. To correct this situation the iron equipment was, for the most part, gradually replaced by copper. It soon developed that copper enhanced the problem and made its solution still more difficult.

Copper clouds occur in certain white wines, particularly those containing free sulfur dioxide, which have been stored in sealed containers for a period of time. Factors affecting cloud formation are wine composition, oxidation-reduction potential of the wine, sunlight, and heat *(10)*. Ribéreau-Gayon has done a considerable amount of pioneering work in this field and originally concluded that the cloud contained tin sulfide *(9)* but later changed his viewpoint and decided that the cloud was mainly copper sulfide. Recently, the mechanism of copper-cloud formation has been examined by Joslyn and Lukton *(4)*.

The precipitates with which Ribéreau-Gayon worked did not contain even a trace of sulfites *(10)*. However, Rentschler and Tanner *(8)* contradicted these findings entirely and maintained that the cloud material was primarily cuprous sulfite.

Although it is generally agreed that the presence of copper is essential for this type of cloud formation, the maximum amount of copper that a wine can tolerate is very controversial. Anywhere from a trace of copper to 100 mg./l. have been reported *(1, 13)* as tolerable without clouding, with the majority of the industry believing that a wine must not contain more than 0.2-0.3 mg./l. of copper to be stable.

Proteins also play an important role in clouding of certain white wines even though they are present in quantities of only 0.01% or less. Ribéreau-Gayon *(11)* reported that one can distinguish between those turbidities that result from the presence of copper and those due to proteins. Upon exposure to air, copper clouds disappear whereas protein clouds persist.

Prevention of copper-cloud formation can be attained by removing the copper ion. The most efficient method of reducing the metal content of wines is "blue-fining" as reported by Fessler *(2)* and many others *(7)*. In "blue-fining," copper and iron are precipitated as the ferrocyanides by the addition of potassium ferrocyanide under conditions controlled to prevent traces of ferrocyanide from remaining in the wine.

---

* Present address, C & H Sugar Company, Crockett, California.

Joslyn, Lukton, and Crane *(5)* have recently finished a survey of 18 organic chelating agents as metal precipitants. Only 3 compounds were found which effectively removed copper from wines by forming insoluble precipitates and two of these were ferrocyanides. The other compound, rubeanic acid, was very effective but has two limitations at the present time; namely, cost and the necessity of adding small amounts of alcohol to the wine as a solvent.

METHODS

Copper. Copper determinations on wine were made by a colorimetric method suggested by Marsh *(6)*, using sodium diethyldithiocarbamate. The copper content of the isolated cloud precipitates was determined by the modified wet-ashing procedure of Coulson-Drabkin *(6)*.

Iron. The total iron content of wine was determined by a colorimetric method using ortho-phenanthroline as described by Saywell and Cunningham *(12)*.

Protein. The protein content of wines was determined by a combination of several procedures. After concentration of 100 ml. of wine to 25 ml. at 40° C. to 45° C. in a vacuum oven, 25 ml. of 25% trichloroacetic acid solution was added and precipitation of the protein was accomplished according to the procedure of Hoch and Vallee *(3)*. Following centrifugation and one washing with 12.5% trichloroacetic acid, the precipitate was analyzed for total nitrogen by the micro-Kjeldahl method. The concentration of protein was then calculated as follows:

$$\text{mg. protein/liter} = \text{mg. N/liter} \times 6.25$$

Phosphorus. The phosphorous content of wines was determined using the ammonium molybdate colorimetric method of Sumner *(15)*.

Sulfur. Sulfur was determined qualitatively by first preparing a sodium fusion of the cloud material following the procedure described by Shriner and Fusion *(14)*. Two drops of a sodium nitroprusside solution were added to a few drops of the clear filtrate and a reddish-violet coloration indicated the presence of sulfur.

The quantitative determination of sulfur was made using the photo-nephelometric micro method of Toennies and Bakay *(16)*.

Measurement of cloudiness. Nephelometer readings were made on a Coleman Nephelometer and are an indication of degree of cloudiness.

Method of desalting the copper-clad hydrolyzates. Preparatory to chromatography all cloud hydrolyzates were desalted using a Reco Electric Desalter.

Manner of forming and isolating copper clouds. Copper clouds were formed by adding the required amount of copper as a standard copper acetate solution to brilliant filtered wines. Nitrogen gas was then bubbled through the wines to help attain reducing conditions. The sealed bottles of wine were then placed in direct sunlight for various periods of time until clouds were formed.

Cloud formation was allowed to proceed until the cloud settled to the bottom of its container. The clear supernatant wine was siphoned off and the cloud isolated from the remaining mixture by centrifugation. The cloud material was then washed with distilled water and dried in a vacuum desiccator over concentrated sulfuric acid. The dried crystalline black material was pulverized in a small agate mortar and stored in stoppered bottles under normal room conditions.

EXPERIMENTAL

Composition of some copper clouds from commercial wines. The composition of two copper clouds and the commercial Sauterne wines from which they were isolated are given in Table 1. The Shriner and Fuson *(14)* test for sulfur on the cloud material was negative, and it appeared that the cloud might be a copper protein.

In order to obtain sufficient cloud material for extensive examination, clouds were induced to form in the laboratory in white table wines containing no added sulfur dioxide. The analysis of the cloud from sample #4 and of the wine before and after

TABLE 1

Composition of copper clouds and the commercial Sauterne wines from which they were isolated

Constituent	Wine		Cloud from wine	
	No. 1, after cloud formed	No. 2, after cloud formed	No.1	No. 2
Total organic nitrogen	312 mg./liter	309 mg./liter	7.94%	10.34%
Protein (total N × 6.25)	1 mg./liter	4 mg./liter	49.6%	64.6%
Copper	4 mg./liter	21 mg./liter	10.8%	21.0%
Sulfur	.....	.....	neg.	neg.
Iron	4.9 mg./liter	3.9 mg./liter	.....	.....
Tannin	0.03%	0.041%	.....	.....
Total SO_2	11 mg./liter	13 mg./liter	.....	.....
Alcohol	10.4%	10.7%	.....	.....
pH	3.65	3.75	.....	.....

clouding is given in Table 2. The total organic nitrogen of wine #4 did not change significantly, but the copper content dropped from 1.1 mg./l. to 0.35 mg./l. From the copper content of the cloud material a minimal molecular weight of around 4000 was calculated for the nitrogenous moiety assuming one molecule of copper for each molecule of copper-protein complex. This again suggested that the cloud material might consist of polypeptides or proteins. Closer examination of the nitrogenous substances seemed logical.

The total organic nitrogen in most wines was found to range from 300 to 600 mg./liter, and of this nitrogen, only 1½%-4% was present in the form of proteins. Therefore, wines contain approximately 0.01% of protein and about 0.50% of free amino acids. In spite of this small protein concentration, it appears to be one of the main causes of cloudiness in white table wines.

Chromatography appeared to offer the simplest method of resolving the nitrogen constituents of the cloud material. After many early failures to obtain an amino acid pattern, even after hydrolysis of the cloud material, it was found that the copper complexes were actually being chromatographed. These complexes would not react with ninhydrin to give the characteristic blue color obtained with amino acids. Therefore, before chromatographing the cloud material, it was necessary to remove the copper from the complex without disrupting the nitrogenous part of the molecule. This was accomplished by shaking the cloud material in a 1% sodium diethyldithiocarbamate solution for several hours and then extracting the resulting copper salt with amyl acetate.

A chromatogram of the water phase failed to show any free amino acids but after alkaline hydrolysis and desalting, a very interesting amino acid picture was obtained showing 12 to 15 different amino acids. This proved quite conclusively the presence of a polypeptide or protein in the cloud material.

Aspartic acid, glutamic acid, serine, glycine, alanine, phenylalanine, and tyrosine were present in the largest amounts, with traces of threonine and proline. A spot cor-

TABLE 2

Composition of wine No. 4 before and after clouding and the clouds isolated therefrom

Constituents	Wine No. 4		Cloud from wine No. 4
	Before clouding	After clouding	
Copper	1.1 mg./liter	0.35 mg./liter	1.64%
Total organic nitrogen	550 mg./liter	547 mg./liter	10.15%
Protein	.....	90 mg./liter	10.15 × 6.25 = 63.4%
Moisture	.....	.....	10.25%
Sulfur	.....	.....	Trace
Total SO_2	9 mg./liter	9 mg./liter	.....
Alcohol	11.4%	11.4%	.....
pH	3.5	3.5	.....

responding to valine or methionine or tryptophane or all three was also present, as was a spot that indicated the presence of one or more of the leucines. Histidine, asparagine, arginine, taurine, β-alanine, and lysine were not observed.

An attempt to remove the copper directly from the cloud by a desalting operation was unsuccessful, indicating that the copper was held quite tenaciously by the nitrogenous constituent.

Several other cloud materials were isolated, employing similar techniques, and each of these contained 10-11% total organic nitrogen, 0.1-17.0% copper, approximately 10% moisture, and traces of sulfur. It was interesting to note that although the protein content of the various copper clouds was relatively constant, i.e., 60-70%, the copper content varied markedly from 0.1 to 21%.

Synthetic wine systems. One approach to the solution of the copper-clouding problem appeared to be through the use of synthetic systems. Because of the very complex nature of wine, it would be practically impossible to duplicate a commercial wine synthetically in the laboratory. However, we could begin to approach the composition of wine as far as the major constituents are concerned. Synthetic wines of various combinations were prepared in one-gallon amounts as follows:

Synthetic Wine Systems

	A	*B*	*C*	*D*
Ethyl Alcohol	15%	15%	15%	15%
Tartaric Acid	0.7%	0.7%	0.7%	0.7%
Tannic Acid	0.05%	0.05%	0.05%	0.05%
Benzoic Acid	0.01%	0.01%	0.01%	0.01%
Copper	4 mg./l.	4 mg./l.	4 mg./l.	0
Lactalbumin Hydrolyzate	0	500 mg. N/l.	500 mg. N/l.	500 mg. N/l.
Total SO_2	250 mg./l.	250 mg./l.	0	0
pH	3.15	3.00	3.10	3.15

All of these wines were filtered after compounding because some of the lactalbumin hydrolyzate was insoluble. After bubbling nitrogen through each wine for ten minutes, the bottles were sealed and placed out-of-doors in the sunlight.

Synthetic wine system "A" remained clear even after exposure to intense sunlight over a two-week period. However, after only 6 hours, wine system "B" formed a cloud which was almost completely reversible when exposed to air. After examination, sample B was resealed and its cloud permitted to form again. After 48 hours of exposure "wine C" formed a partially reversible cloud. After 72 hours "wine D" formed a slight cloud which was irreversible when exposed to air.

Samples B, C, and D all showed cloud formation but they differed from one another. Cloud D was probably a protein-tannin cloud being completely irreversible and having been formed in a system that did not contain any copper or sulfur dioxide. Cloud C was partially reversible and probably contained a copper-polypeptide in addition to the protein-tannin cloud postulated in "wine D." Cloud B is undoubtedly similar to Cloud C with the sulfur dioxide acting as a catalyst hastening conditions necessary for cloud formation. It is also believed that the nitrogen source of lactalbumin hydrolyzate might help conditions necessary for a copper sulfide cloud in addition to the clouds already postulated.

No clouding occurred in "wine A" which contained all the added ingredients except a nitrogen source, indicating the importance of certain nitrogenous substances for cloud formation.

Synthetic wines B, C, and D were assayed for protein and were found to contain approximately 1.7 mg. of protein nitrogen, which is equivalent to 11 mg. of protein per liter of wine. The total nitrogen content of wines B, C, and D was 507 mg. per liter.

Additional synthetic wines were prepared to check other combinations that might possibly cause cloud formation. The series contained wines having the following compositions:

Synthetic Wine Systems

	E	F	G	H
Ethyl Alcohol	15%	15%	15%	15%
Tartaric Acid	0.7%	0.7%	0.7%	0.7%
Tannic Acid	0	0	0.05%	0.05%
Benzoic Acid	0.01%	0.01%	0.01%	0.01%
Lactalbumin Hydrolyzate	500 mg. N/l.	500 mg. N/l.	500 mg. N/l.	500 mg. N/l.
Copper	4 mg./l.	0	4 mg./l.	4 mg./l.
Total SO_2	0	250 mg./l.	250 mg./l.	250 mg./l.
pH	3.05	3.10	4.00	5.10

After thirty hours "wine E" formed a slightly reversible cloud whereas "wine F" failed to form a cloud even after several months' storage. Synthetic wines G and H differed from synthetic wine B only in pH. Samples G and H formed clouds of much less intensity and considerably slower than did "wine B," indicating that pH has a marked effect on cloud formation. The clouds that formed in the synthetic wines were removed as described earlier and analyzed for copper and total nitrogen (see Table 3).

TABLE 3

Composition of clouds obtained from the various synthetic wines

	B	C	D	E	G	H
	per cent					
Copper	49.9	14.8	0.5	27.5	26.6	26.3
Total nitrogen	2.1	4.8	5.0	6.3	2.8	2.3
Protein = (N × 6.25)	13.1	30.0	31.3	39.4	17.5	14.4

Synthetic wines B, G, and H, which contained all the ingredients used, yielded clouds with high copper sulfur and relatively low protein contents. Synthetic wine B, which most closely resembled a natural wine system, yielded a cloud containing approximately 15% sulfur. Synthetic wines C, D, and E, on the other hand, did not contain sulfur dioxide and yielded clouds having significantly higher protein contents.

Sample D was the only "wine" to form an irreversible cloud and, likewise, it was the only cloud-forming wine that did not contain copper. From this series of experiments it may be concluded that copper is necessary for the formation of a reversible cloud. "Wine C" formed a cloud that was partially reversible, indicating that sulfur dioxide is not absolutely necessary for the formation of a reversible cloud. However, the rapidity of cloud formation in "wine B" indicates that sulfur dioxide does enter into cloud formation and can cause a reversible cloud, but not in the absence of proteinaceous or other nitrogenous material as demonstrated in "wine A."

Lactalbumin hydrolyzate was necessary for cloud formation in all synthetic wines, but the fraction of the hydrolyzate responsible for cloud formation remained undetermined. Whether amino acids would serve as the nitrogen source responsible for clouding appeared doubtful, but samples were prepared to test this possibility.

Samples of synthetic wines B, I, and J were compared. "Wines I" and "J" were identical with "wine B" except the glutamic acid (150 mg. N/liter) and glycine (150 mg. N/liter) were substituted for lactalbumin hydrolyzate, and in the case of "wine I" tannic acid was omitted. All of the "wines" had a pH of 3.

In the sunlight "wine B" clouded within a few hours while "wines I" and "J" remained brilliant for more than two weeks. Duplicate samples of B and I were stored in the dark in a 45° C. incubator to check the effect of sunlight on cloud formation. After one week, sample B was still brilliant but then clouded very slowly until after 4 months, when a heavy black precipitate formed. "Wine I" was still brilliant after two months' storage.

The rapid clouding of "wine B" in the sunlight, in contrast to samples I and J, probably indicates that the small amount of polypeptide and protein present in "wine B" either became part of the cloud material, catalyzed clouding by entering into oxidation-reduction reactions, or participated in both of these reactions. Although

"wine B" clouded in the dark at 45° C., it required considerably more time to do so than when stored in the sunlight. This demonstrated the very important catalytic action of sunlight in cloud formation and that this action is more than just a heat effect.

DISCUSSION AND CONCLUSIONS

Copper clouds isolated from commercial wines were found to be high in nitrogen and relatively low in sulfur content. This suggests that the cloud was a copper-protein complex on which other minor undetermined constituents were absorbed. Chromatographic analysis of the cloud material confirmed the fact that the nitrogenous constituent of the clouds was proteinaceous in nature rather than free amino acids.

Synthetic wine systems were prepared to establish the factors responsible for the copper-cloud formation. Lactalbumin hydrolyzate was used in the synthetic wines because it should come close to duplicating the nitrogen conditions in commercial wine, i.e., high nitrogen but low protein content. No clouding occurred in synthetic systems when a source of nitrogen was absent or when only amino acids were present.

Copper was necessary for the formation of a reversible cloud in synthetic systems. Sulfur dioxide was not necessary for the formation of a reversible cloud, although when present with copper and protein a partially reversible cloud resulted. Sulfur dioxide always catalyzed cloud formation in the sunlight whenever copper and protein were present. It undoubtedly hastens the development of reducing conditions and enters as well into the cloud material as copper sulfide. A completely irreversible cloud would form if copper and sulfur dioxide were absent, whereas a partially reversible cloud would form if copper was present but not sulfur dioxide. If both copper and sulfur dioxide were present, an almost completely reversible cloud would form initially, but would become progressively more irreversible with time. These results indicate that various types of clouds may form independently and simultaneously under the different conditions.

It is concluded that what is commonly referred to as a copper cloud is really a combination of several clouds, primarily protein-tannin, copper-protein, and copper-sulfur complex (probably copper sulfide).

LITERATURE CITED

1. FESSLER, J. H. Development of the Fessler compound. *Wines and Vines*, **33**, 15 (1952).
2. FESSLER, J. H. The present status of blue-fining. *Fruit Products J.*, **28**, 167 (1949).
3. HOCH, L. H., AND VALLEE, B. L. Gravimetric estimation of proteins precipitated by trichloroacetic acid. *Anal. Chem.*, **25**, 317 (1953).
4. JOSLYN, M. A., AND LUKTON, A. Mechanism of copper casse formation in white table wines. (Submitted for publication.)
5. JOSLYN, M. A., LUKTON, A., AND CRANE, A. The removal of excess copper and iron from wine. *Food Technol.*, **7**, 21 (1953).
6. MARSH, G. L. Copper Determination, Cited in Joslyn, M. A., and Amerine, M. A. Commercial Production of Dessert Wines. Calif. Agr. Expt. Sta. *Bull. 651*, 163-166 (1941).
7. MARSH, G. L. Report on the Fessler compound. *Wines and Vines*, **33**, 19 (1952).
8. RENTSCHLER, H., AND TANNER, H. Concerning the Copper-Sulfite Haziness in White Wines and Unfermented Juices. *Schweitz. Z. für Obst und Weinbau*, **60**, 298-301 (1951).

9. RIBÉREAU-GAYON, J. Concerning certain accidental cloudings of white wines. *Proc. Verbaux de la Societe des Sciences Physiques et Naturelles de Bordeaux*, Jan. 12, 1928, and *Revue de Viticulture*, Jan., 1928.
10. RIBÉREAU-GAYON, J. Contribution a l'etude des oxydations et reductions dans les vins. *Application a l'Etude du Vieillissement et des Casses.* 2nd ed., 1933. Delmas, Bordeaux.
11. RIBÉREAU-GAYON, J. *Traite d'Oenologie.* Paris, Librairie Polytechnique Ch. Beranger, 1947.
12. SAYWELL, L. G., AND CUNNINGHAM, B. Determination of iron. *Ind. Eng. Chem., Anal. Ed.*, **9**, 67-69 (1937).
13. SEARLE, H. B., LAQUE, F. L., AND DONROW, R. H. Metals and wines. *Ind. Eng. Chem.*, **26**, 617-627 (1934).
14. SHRINER, R. L., AND FUSON, R. C. *The Systematic Identification of Organic Compounds.* 1935, John Wiley and Sons, Inc., New York, N. Y.
15. SUMNER, J. B. A method for the colorimetric determination of phosphorus. *Science*, **100**, 413 (1944).
16. TOENNIES, G., AND BAKAY, B. Photonephelometric micro-determination of sulfate and organic sulfur. *Anal. Chem.*, **25**, 160-165 (1953).

HYDROLYSIS OF PECTIC MATERIALS AND OLIGOURONIDES BY TOMATO POLYGALACTURONASE[a]

B. S. LUH, S. J. LEONARD, AND H. J. PHAFF
Department of Food Technology, University of California, Davis, California

(Manuscript received February 6, 1956)

Two general types of pectic enzymes are involved in the breakdown of pectic materials: pectin esterase (PE), which splits the methyl ester linkages in pectinic acid or pectin to yield pectic acid, and polygalacturonase (PG), which hydrolyzes the 1,4-alpha-glycosidic bonds in the uronide chain to yield galacturonic acid. The existence of more than one kind of polygalacturonase has received increased attention in recent years *(1, 2, 3, 12, 20, 22, 23, 24)*.

Pectin-degrading enzymes in tomatoes have been studied by several workers *(10, 11, 12, 13, 14, 19, 21)*. McColloch and Kertesz *(13)* reported that the maximum increase in reducing value of pectic acid acted upon by tomato depolymerase is about 50% of the theoretically possible value. It is difficult, however, to correlate the presumed end-product of the reaction at 50% hydrolysis, a postulated pentamer of galacturonic acid, with such a high percentage of glycosidic hydrolysis.

Roelofsen *(21)* reported that crude tomato extract does not contain a pectic acid depolymerase, but a polygalacturonase able to split pectic acid into a mixture of mono-, di-, and trigalacturonic acids. The most recent work on this enzyme by McCready, McComb, and Jansen *(14)* shows that tomato PG can hydrolyze pectic acid completely to galacturonic acid. In this respect, it is qualitatively similar to purified fungal PG, although quantitatively the tomato enzyme showed a slower rate of digalacturonic acid hydrolysis than the fungal enzyme. In view of recent interest in the nature and complexity of polygalacturonase from different sources, and the important role which pectic enzymes play in the consistency of macerated tomato products, we have investigated the viscometric changes of pectic acid and the rate, degree, and products of hydrolysis of pectic acid by pectic enzyme preparations from Pearson or San Marzano tomatoes. In addition, the action of a partially purified tomato PG on di-, tri-, and tetragalacturonic acids was also studied.

EXPERIMENTAL

Extraction of pectic enzymes. The method for extraction of pectic enzymes from tomatoes was the same as that described by McColloch, Keller, and Beavens *(10)*. Firm ripe Pearson and San Marzano tomatoes were macerated in a Waring blender for one minute at room temperature with an equal weight of 10% sodium chloride solution. The resulting mixture was filtered through Whatman No. 1 filter paper, and the filtrate was stored in the frozen condition at −12° C.

Precipitation of the crude enzyme by ammonium sulfate. An aliquot of 250 ml. of the crude sodium chloride extract was cooled to a temperature between 2 and 5° C.

[a] Presented in part at the 14th Annual Meeting of the Institute of Food Technologists, Los Angeles, California, June, 1954.

Then, 159 g. of ammonium sulfate was slowly added while stirring. The precipitated enzyme was centrifuged in a Servall centrifuge for 20 minutes at 4° C. After decanting the supernatant liquid the precipitated enzyme was dissolved in 25 ml. of 0.05 M acetate buffer at pH 4.5. The resulting enzyme solution was dialyzed in the cold in a rotating Visking bag against 0.05 M acetate buffer at pH 4.5, until the dialysate was free of sulfate ions. After dialysis the volume of enzyme solution was made up to 50 ml.

Determination of PG activity. Polygalacturonase activity was determined by following the increase in reducing groups by the modified Willstäter-Schudel hypoiodite method as described by Jansen and MacDonnell *(6)*, except that the reaction was carried out at pH 4.5. The reaction mixture consisted of 50 ml. of 1.0% Wichmann pectic acid at pH 4.5, 20 ml. of 0.5 M acetate buffer, pH 4.5, the proper amount of enzyme solution, and distilled water to make 100 ml. All reactions were carried out at 29.4 ± 0.1° C. Wichmann pectic acid was prepared according to Newbold and Joslyn *(16)*. Di- and trigalacturonic acids were prepared by the procedure of Phaff and Luh *(18)* and tetragalacturonic acid by the method of Demain and Phaff *(5)*.

For determining relative activities of tomato PG on different oligouronides and Wichmann pectic acid, a semi-micro method based on the same principle as the Willstätter-Schudel hypoiodite method was used. In this case the substrate consisted of 0.125 g. of the oligouronic acid dissolved in 10 ml. of distilled water. Next 1 N NaOH was added slowly, during stirring, until the pH value reached 4.5. To the resulting solution was added 5 ml. of 0.5 M acetate buffer at pH 4.5 and sufficient distilled water to make the final volume 20 ml. Then 5 ml. of the concentrated tomato PG was added. Aliquots of 2 ml. samples were removed at certain time intervals and pipetted into a 25-ml. glass-stoppered Erlenmeyer flask containing 0.35 ml. of 2 M Na_2CO_3. Then 2 ml. of 0.1 N iodine were added and the reaction mixture was allowed to stand for exactly 20 minutes counted from the addition of iodine. Then 0.8 ml. of 2 M H_2SO_4 was added and the excess iodine titrated with 0.025 N $Na_2S_2O_3$. One unit of PG activity (PG_u) is that amount of enzyme which liberates one millimole of aldehyde groups per minute at the temperature indicated.

The decrease in viscosity of pectic acid due to PG activity was also followed in an Ostwald viscosimeter at 29.4 ± 0.1° C. The Ostwald viscosimeter reading for distilled water was 77.8 seconds.

Pectin esterase activity. This was assayed by measuring the rate of liberation of carboxyl groups of pectin N.F. by the method of MacDonnell *et al. (9)* at pH 7.5 and a temperature of 29.4 ± 0.1° C. in the presence of 0.5% sodium chloride. The extension electrodes of a Coleman pH meter were used to check the pH. A blank sample was run in which the added enzyme had been previously inactivated by placing it in a boiling water bath for 10 minutes. One unit of pectin esterase activity (PE_u) is that amount of enzyme which releases one milliequivalent of carboxyl groups per minute.

Paper chromatography. The oligouronides formed by tomato PG during the hydrolysis of Wichmann pectic acid were separated on Whatman No. 4 filter paper by the modified method of Jermyn and Isherwood as described by Demain and Phaff *(5)*. The sample was first treated with Duolite C-3 cation exchange resin to remove metallic ions prior to chromatography.

RESULTS AND DISCUSSION

Polygalacturonase and pectin esterase activity in Pearson and San Marzano tomatoes. PG and PE activity in extracts from firm ripe tomatoes are shown in Table 1. It can be seen that the activities of the pectic enzymes in the two varieties of tomato are roughly comparable on the fresh basis. Since Luh *et al. (7)* found that the total solids content of the two varieties of tomatoes in the firm ripe condition was not greatly different, the comparison on the fresh basis appears valid. The level of PE activity in the tomato extracts was 23 to 27 times higher than that of the PG. However, PE from tomato was shown by McColloch and Kertesz *(11)* to be nearly inactive or much less active at pH 4.5 than at 7.5, even if 0.1 M NaCl was used as activator. Since activity of PG from tomato was assayed

TABLE 1

Pectic enzyme activity in firm ripe tomatoes. The crude NaCl extracts of the tomatoes were used as enzyme solutions

Variety	Polygalacturonase activity (PG_u) g. of fresh fruit	Pectinesterase activity (PG_u) g. of fresh fruit	Ratio $(PE_u)/(PG_u)$
Pearson	1.89×10^{-3}	4.40×10^{-2}	23.2
San Marzano	1.61×10^{-3}	4.40×10^{-2}	27.3

at pH 4.5, whereas that of PE was assayed at 7.5, the ratio of $(PE_u)/(PG_u)$ presented in Table 1 may not represent the actual activity in macerated tomatoes which do not contain salt and are usually in the pH range between 4.2 and 4.5.

Change in reducing value and viscosity. When a 0.5% solution of pectic acid at pH 4.5 is treated with tomato PG and the decrease in viscosity is correlated with the increase in glycosidic hydrolysis, a relationship is found which strongly suggests a random type of hydrolysis of pectic acid. An example is shown in Figure 1. A rapid initial lowering of the viscosity is accompanied by only a small increase in reducing value of the reaction mixture. Four per cent of the glycosidic bonds was hydrolyzed at the time when a 50% drop in viscosity had occurred (9 minutes). It was previously shown *(8)* that the weight-average molecular weight of the Wichmann pectic acid used for the experiment was about 24,500.

Hydrolysis of Wichmann pectic acid by tomato PG. Hydrolysis of Wichmann pectic acid by a dialyzed ammonium sulfate precipitated enzyme preparation from the Pearson variety is illustrated in Figure 2. The course of hydrolysis consists of two distinct phases, (a) an initial rapid stage in which the increase in reducing value is a linear function of time and, (b) a second stage during which the rate of hydrolysis becomes progressively slower. At the end of the first phase, a rather sharp break in the curve will be noted after about 25% hydrolysis has occurred. After

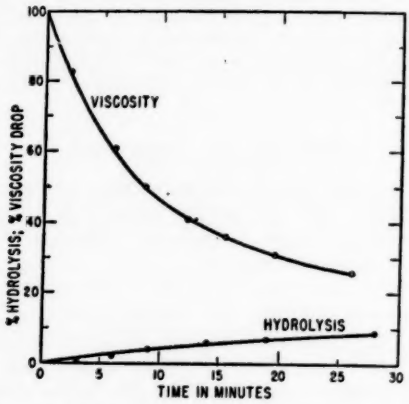


Figure 1. Relation between viscosity change and glycosidic hydrolysis of pectic acid by tomato PG at pH 4.5.

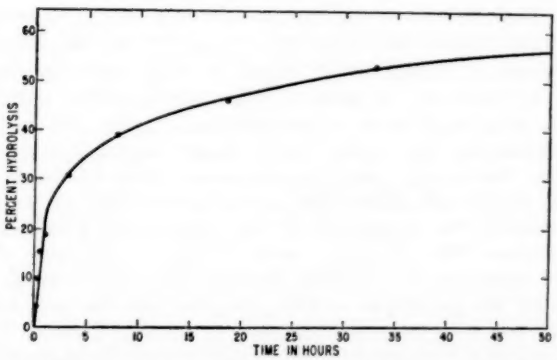


Figure 2. Hydrolysis of 0.5% sodium pectate (pH 4.5) by a partially purified preparation of tomato PG. Enzyme concentration 20% v/v. Per cent hydrolysis based on complete hydrolysis to galacturonic acid as 100%.

50 hours the hydrolysis was still continuing and reached 61.3% after 69 hours and 71.7% after 118 hours. In another experiment, 85% hydrolysis was found after 3 weeks' incubation with toluene as a preservative. In control samples containing heat-inactivated tomato PG, no increase in reducing value was found after 7 days.

If pectic acid is hydrolyzed by a random mechanism, as was indicated by the viscometric changes due to the action of tomato PG, the initial rapid rate consists in all likelihood of a splitting of the large polyuronide fragments into smaller units. Since the break occurs at 25% hydrolysis, the average size of the oligouronides present at this point must be approximately that of a tetramer.

Chromatography of the products of hydrolysis. Gross changes during the course of action of tomato PG on pectic acid can also be followed by chromatographing the various intermediate fractions during the course of

TABLE 2

Paper chromatographic analysis of galacturonides. Hydrolysis of 0.5% pectic acid by tomato polygalacturonase at pH 4.5. Enzyme concentration 0.018 PG_u per 100 ml. of reaction mixture

Duration of enzymatic hydrolysis	Galacturonides						Total galacturonic bonds split
	Poly-	Penta-	Tetra-	Tri-	Di-	Mono-	
Hr.							%
0	+	−	−	−	−	−	0
0.5	+	+	+	+	(+)[1]	−	15.4
1.0	+	+	+	+	(+)[1]	−	18.8
3.2	(+)[1]	+	+	+	+	(+)[1]	30.7
8.9	−	(+)[1]	+	+	+	+	39.2
18.5	−	−	+	+	+	+	46.2
33.0	−	−	(+)[1]	+	+	+	53.0
69.0	−	−	−	+	+	+	61.3
118.0	−	−	−	(+)[1]	+	+	71.7
380.0	−	−	−	−	+	+	80.0

[1] (+) represents weak spots.

hydrolysis (Table 2). Since a chromatogram does not detect intermediates or end-products present in very small concentrations, the exact times of their appearance or disappearance cannot be read from the paper. However, important clues may be obtained regarding the course of the reaction. During the initial rapid phase of the hydrolysis, pectic acid appears to be broken progressively into smaller units, finally resulting in a mixture in which penta-, tetra-, tri-, and digalacturonic acids predominate, while galacturonic acid is still absent. The second slower stage of the hydrolysis is characterized by the appearance of free galacturonic acid and the gradual disappearance first of penta-, next of tetra-, and finally of trigalacturonic acid, resulting in a mixture of mono- and digalacturonic acid. At this last point the hydrolysis is 80% complete and the spot for digalacturonic acid was noticeably weaker than at previous points. The results also show that at a stage between 50 and 60% hydrolysis, mono-, di-, and trigalacturonic acids are the only breakdown products present. These findings are in good agreement with those of McCready *et al. (14)*.

Hydrolysis of oligogalacturonides. Successive disappearance of the penta-, tetra-, and trigalacturonic acids in the order of their decreasing galacturonide chain lengths indicated possible differences in their rates of hydrolysis by tomato PG. This was also indicated by the continually slower rate of hydrolysis of pectic acid after the first rapid stage. Hydrolysis of oligouronides also gives information about the true end-products of the hydrolysis of pectic acid by tomato PG. Demain and Phaff *(3)* have shown that yeast polygalacturonase hydrolyzes pectic acid to a mixture of di- and monogalacturonic acid, whereas it is well known that fungus PG splits pectic acid completely into galacturonic acid. Clear proof as to the final hydrolysis product can be obtained most directly by studying the action of the enzyme preparation on digalacturonic acid. In order to test the above indications experimentally, 0.5% solutions of di-, tri-, and tetragalacturonic acids were treated at pH 4.5 with tomato PG (0.018 PG_u per 100 ml. reaction mixture). The increases in reducing values were determined periodically and the results are plotted in Figure 3. For comparison the same reaction was followed also with pectic acid as the substrate. The results clearly show that the initial rate of hydrolysis decreases by lowering the galacturonide chain length. If the initial rate with pectic acid is called 100, those of tetra-, tri-, and digalacturonic acid are approximately 7.0, 1.58, and 1.05%, respectively. It is difficult to estimate these ratios accurately since the hydrolysis curves, in particular those of the dimer and the trimer, are only linear during a very limited percentage of their total hydrolysis (less than 10%). Possibly, some of the hydrolysis products partially inhibited the activity of tomato PG on the higher oligouronides, as was demonstrated by Demain and Phaff *(4)* for yeast PG acting on tetragalacturonic acid. McCready and Seegmiller *(15)* found that purified fungal polygalacturonase hydrolyzed di- and trigalacturonic acids at a rate of approximately 5% of the initial rate on pectic acid. McCready, McComb, and Jansen *(14)* stated that the rate on digalacturonic acid (and possibly trigalacturonic acid) by tomato PG was less than that of fungal enzyme. Our results are in agreement with this. On the other hand, yeast PG hydrolyzes tetra- and trigalacturonic acid much more

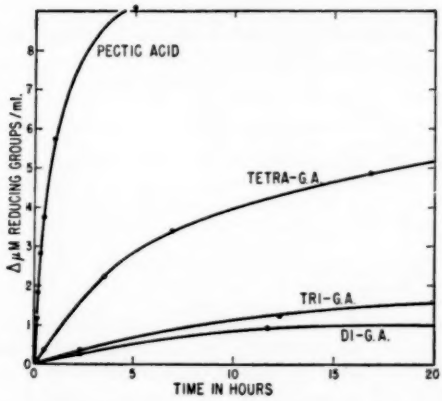


Figure 3. Initial rates of hydrolysis of pectic acid, tetra-, tri-, and digalacturonic acids by tomato PG at pH 4.5.

slowly than either fungal or tomato PG and does not attack digalacturonic acid *(4, 17)*. Our data explain, at least in part, why the second stage of hydrolysis of pectic acid by tomato PG is very slow. It can be seen that in particular digalacturonic acid was broken down at an extremely low rate with the enzyme preparations available. In order to confirm the splitting of the dimer by tomato PG, a series of chromatograms were run of such reaction mixtures after various periods of time. Toluene was used as a preservative. Intensity of the galacturonic acid spots was found to increase with time and those of digalacturonic acid to decrease. After 5 days, the latter compound gave only a relatively weak spot whereas galacturonic acid was very distinct. A control sample with boiled enzyme showed only a spot for digalacturonic acid.

Finally, the hydrolysis of tetragalacturonic acid was followed by paper chromatography. The purpose of this experiment was to obtain information as to the mechanism of splitting. Demain and Phaff *(4)* demonstrated that yeast polygalacturonase splits tetragalacturonic acid into the trimer and the monomer (3 + 1 split), which is followed by a slower reaction in which trigalacturonic acid is hydrolyzed into the dimer and monomer. In other words, yeast polygalacturonase does not split tetragalacturonic acid at a detectable rate into two moles of digalacturonic acid. These authors explained this observation by the fact that tetragalacturonic acid is split about 50 times as fast as the trimer and the central bond in the tetramer is the corresponding bond which is hydrolyzed in the trimer.

The results with tomato PG are presented in Table 3. It can be seen that after a very short time primarily mono- and trigalacturonic acids are formed indicating a preferred (3 + 1) split. However, subsequent samples show all three of the lower uronides, until the trimer finally disappears. The early appearance of digalacturonic acid in the case of tomato PG as compared to its absence in the case of yeast polygalacturonase can be explained by considering the rates of hydrolysis of trigalacturonic acid

TABLE 3

Hydrolysis of tetragalacturonic acid at pH 4.5 by tomato PG as shown by paper chromatography

Time, hr.	Galacturonides			
	Tetra-	Tri-	Di-	Mono-
0	+	−	−	−
0.3	+	+	(+)[1]	+
5.5	(+)[1]	+	+	+
16.3	−	+	+	+
67.0	−	−	+	+
118.0	−	−	+	+

[1] (+) represents weak spots.

by the two enzyme systems. The ratio of hydrolysis rates of tetra- to trigalacturonic acid is about 50 for yeast polygalacturonase but only 4.5 for tomato PG. The result is that trigalacturonic acid formed from the tetramer by tomato PG is hydrolyzed much more rapidly (2 + 1 split) than it would be by yeast PG, thus accounting for the simultaneous presence of all three of the lowest oligouronides.

SUMMARY

The action has been studied of partially purified preparations of tomato polygalacturonase on pectic acid and on the oligouronides tetra-, tri-, and digalacturonic acids. The reactions were followed by paper chromatography and the results were correlated with the increase in reducing groups. The first 25 to 30% hydrolysis of pectic acid consists of a rapid linear phase during which a random cleavage of the substrate occurs and oligouronides are produced with the exception of free galacturonic acid. The reaction then slows down continually, while galacturonic acid accumulates. At 50% hydrolysis tri-, di-, and galacturonic acids are the products and at 80% only di- and monogalacturonic acid are found. Digalacturonic acid is hydrolyzed very slowly by our preparations. If the initial reaction velocity with pectic acid is taken as 100, those with tetra-, tri-, and digalacturonic acids are approximately 7.0, 1.58, and 1.05, respectively. The preferred attack on tetragalacturonic acid is a cleavage into galacturonic and trigalacturonic acid. The PG and PE content of Pearson and San Marzano tomatoes were found to be approximately equal and the PE activity (at pH 7.5) was about 23 to 27 times as great as the PG activity (at pH 4.5) in the two varieties.

LITERATURE CITED

1. Ayres, A., Dingle, J., Phipps, A., Reid, W. W., and Solomons, G. L. Enzymic degradation of pectic acid and the complex nature of polygalacturonase. *Nature*, **170**, 834 (1952).
2. Brooks, J., and Reid, W. W. The complex nature of polygalacturonase from *Aspergillus foetidus* Thom and Raper. *Chemistry & Industry*, 325-326 (1955).
3. Demain, A. L., and Phaff, H. J. Composition and action of yeast polygalacturonase. *Nature*, **174**, 515 (1954).
4. Demain, A. L., and Phaff, H. J. Hydrolysis of the oligogalacturonides and pectic acid by yeast polygalacturonase. *J. Biol. Chem.*, **210**, 381-393 (1954).
5. Demain, A. L., and Phaff, H. J. The preparation of tetragalacturonic acid. *Arch. Biochem. Biophys.*, **51**, 114 (1954).

6. JANSEN, E. F., AND MACDONNELL, L. R. Influence of methoxyl content of pectic substances on the action of polygalacturonase. *Arch. Biochem.*, **8**, 97 (1945).
7. LUH, B. S., LEONARD, S., AND DEMPSEY, W. Pectic substances of Pearson and San Marzano tomatoes. *Food Research*, **19**, 146-153 (1954).
8. LUH, B. S., AND PHAFF, H. J. End products and mechanism of hydrolysis of pectin and pectic acid by yeast polygalacturonase. *Arch. Biochem. Biophys.*, **51**, 102 (1954).
9. MACDONNELL, L. R., JANSEN, E. F., AND LINEWEAVER, H. The properties of orange pectinesterase. *Arch. Biochem.*, **6**, 389-401 (1945).
10. MCCOLLOCH, R. J., KELLER, G. J., AND BEAVENS, E. A. Factors influencing the quality of tomato products. I. Surface-localized pectic enzymes inactivated by blanching. *Food Technol.*, **6**, 197-199 (1952).
11. MCCOLLOCH, R. J., AND KERTESZ, Z. I. Pectic enzymes. VIII. A comparison of fungal pectin-methylesterase with that of higher plants, especially tomatoes. *Arch. Biochem.*, **13**, 217-229 (1947).
12. MCCOLLOCH, R. J., AND KERTESZ, Z. I. An unusually heat-resistant pectolytic factor from tomatoes. *Arch. Biochem.*, **17**, 197-199 (1948).
13. MCCOLLOCH, R. J., AND KERTESZ, Z. I. Recent developments of practical significance in the field of pectic enzymes. *Food Technol.*, **3**, 94-96 (1949).
14. MCCREADY, R. M., MCCOMB, E. A., AND JANSEN, E. F. The action of tomato and avocado polygalacturonase. *Food Research*, **20**, 186-191 (1955).
15. MCCREADY, R. M., AND SEEGMILLER, C. G. Action of pectic enzymes on oligogalacturonic acids and some of their derivatives *Arch. Biochem. Biophys.*, **50**, 440-450 (1954).
16. NEWBOLD, R. P., AND JOSLYN, M. A. Chemistry of analytically important pectic acids. I. Chemical properties of Ehrlich, Wichmann and Carre-Haynes pectic acids. *J. Assoc. Offic. Agric. Chem.*, **35**, 872-891 (1952).
17. PHAFF, H. J., AND DEMAIN, A. L. The uni-enzymatic nature of yeast polygalacturonase. *J. Biol. Chem.*, **218**, 875-884 (1956).
18. PHAFF, H. J., AND LUH, B. S. The preparation of pure di- and trigalacturonic acids. *Arch. Biochem. Biophys.*, **36**, 231-232 (1952).
19. PILNIK, W., AND ROTHSCHILD, G. Pectolytic enzymes in tomatoes. *Bull. Res. Counc. Israel*, **4**, 63-65 (1954).
20. ROBOZ, E., BARRATT, R. W., AND TATUM, E. L. Breakdown of pectic substances by a new enzyme from Neurospora. *J. Biol. Chem.*, **195**, 459-471 (1952).
21. ROELOFSEN, P. A. Polygalacturonase activity in yeast, Neurospora, and tomato extract. *Biochem. et Biophys. Acta.*, **10**, 410-413 (1953).
22. SAITO, H. Pectic glycosidases of *Aspergillus niger*. *J. Gen. Appl. Microbiol.*, **1**, 38-60 (1955).
23. SCHUBERT, E. Die trennung der pektinglykosidasen (PG) aus *Aspergillus nigher* durch selecktive inaktivierung und adsorption. *Biochem. Z.*, **323**, 78-88 (1952).
24. SEEGMILLER, C. G., AND JANSEN, E. F. Polymethylgalacturonase, an enzyme causing the glycosidic hydrolysis of esterified pectin substances. *J. Biol. Chem.*, **195**, 327-336 (1952).

MECHANISM OF COPPER CASSE FORMATION IN WHITE TABLE WINE. II. TURBIDIMETRIC AND OTHER PHYSICO-CHEMICAL CONSIDERATIONS

A. LUKTON AND M. A. JOSLYN
Food Technology Department, University of California, Berkeley

(Manuscript received February 10, 1956)

The problem of copper casse formation in wines has been studied intensively both here and abroad *(3, 4, 7, 8, 9, 14, 15, 16, 17, 25, 26)* since its first recognition by Carles in 1908 *(3)*. It has been ascribed to formation of cuprous sulfite *(8, 25)*, mixed cuprous and cupric sulfites *(20)*, copper polypeptides *(17)*, and cupric sulfide *(26)*. On the basis of chemical equilibrium considerations *(21)* the most plausible theory was that proposed by Ribéreau-Gayon *(26)*.

The mechanism of copper casse formation as given by Ribéreau-Gayon *(26)* was not unequivocably demonstrated. He assumed the reactions which may take place to be as follows:

1) $Cu^{++} + RH \rightarrow Cu^{+} + R + H^{+}$ (Reduction of cupric ions)
2) $6\,Cu^{+} + 6\,H^{+} + SO_2 \rightarrow 6\,Cu^{++} + H_2S + 2\,H_2O$ (Reduction of sulfur dioxide)
3) $Cu^{++} + H_2S \rightarrow CuS + 2\,H^{+}$ (Formation of insoluble cupric sulfide)
4) $CuS \xrightarrow[\text{Electrolytes, Colloids}]{\text{Flocculation}}$ (Haze formation)
5) $6\,RH + SO_2 \rightarrow 6\,R + H_2S + 2\,H_2O$ (Sum of reactions (1) and (2) by multiplying (1) by (6).
6) $CuS + 2\,O_2 \rightarrow CuSO_4$ (On exposure to air)

Ribéreau-Gayon assumed copper functions as a catalyst in reaction *(5)* since he did not believe that cupric ion was directly involved in the reduction of sulfur dioxide. He also postulated that the unknown reducing compound, RH, could not directly reduce sulfur dioxide, but would reduce cupric to cuprous ions which in turn reduced sulfur dioxide, thus regenerating cupric ions; finally, the cupric ions reacted with hydrogen sulfide to form cupric sulfide.

In the data presented here, the roles of sulfur dioxide, protein, and several reducing agents in copper casse formations were examined. Some physical and chemical properties of the precipitate were investigated.

EXPERIMENTAL

The generally used experimental methods are given here; the particular conditions used in various phases of investigations are given in the pertinent sections.

Turbidimetry. The wines to be tested were brought to room temperature, filtered if not clear, adjusted to the chemical component concentration desired, and saturated with air to insure a constant oxygen content. They then were stored in 4-ounce, wide-mouth, soft-glass jars which were crown-capped after filling.

Turbidity changes were measured in a turbidimeter, designed to accommodate the jars, as previously described *(14, 16, 21)*. All measurements were taken against a distilled water reference standard set at zero turbidity.

Types and composition of wines. The wines used were selected to provide wide variation in composition, particularly in protein and sulfur dioxide content. Their composition is shown in Table 1. Wine 1, a commercial wine high in sulfur dioxide and low in protein, showed a notable tendency to form a copper casse. Wines 2 through 5 contained larger quantities of protein but were prepared experimentally with minimal quantities of sulfurous acid.

Analytical methods. The alcohol, sugar, acid, extract, tannin, phosphate, sulfur dioxide, total copper and iron content were determined by procedures described previously *(14, 16)*. Sulfate was determined by the usual gravimetric barium sulfate precipitation procedure after expelling sulfur dioxide from the acidified wine by heating in presence of carbon dioxide. Protein was determined as the 10% trichloracetic acid precipitable nitrogenous matter. The wine, after addition of trichloracetic acid was heated at 90° C. for 15 minutes, centrifuged, and the sediment washed twice with 10% trichloracetic acid *(13)*. The nitrogen content of the precipitate was determined by the micro-Kjeldahl method *(2)*.

RESULTS

Factors Affecting Turbidity Changes

Relations between changes in copper content and turbidity. The increase in turbidity with time, usually observed, may be due to the occurrence of a rapid reaction with subsequent agglomeration of particles or to the continuous formation of measurable new particles. If the latter were true, then copper should be incorporated into the precipitate throughout the entire range of turbidity formation and a gradual depletion in the copper content in the supernatant wine would occur. This was investigated in two ways: by electron microscope observations of the precipitate; and by the simultaneous measurement of changes in turbidity and copper content.

Procedure. Wine 1 was adjusted to 5 mg./liter of copper and introduced into a number of sample jars which were capped after sweeping out the headspace with nitrogen. The turbidity of the samples, which were stored in an incubator at 45° C., was measured periodically. A considerable range in turbidity intensity was observed among the samples throughout the run, increasing during the latter stages to about plus or minus 10% about the mean value. The sample close to the mean was chosen for analysis. The precipitates were isolated and washed twice with water by ultracentrifugation. The supernatant wine and precipitate were analyzed for copper. A small measured portion of the precipitate was saved for electron microscope study. After 18 days, when the reaction was practically complete, the remaining samples were opened and saturated with air by vigorous shaking. Copper content and turbidity were periodically determined during the subsequent clarification.

The changes occurring during the formation and solution of precipitate are given in Table 2.

The data presented in Table 2 indicate that a steady increase in copper content of the precipitate occurred as the turbidity increased and the reverse was true during precipitate solution. The data demonstrate a regular correlation between turbidity and copper changes, but the observed turbidity was not directly proportional to copper decrease throughout. More information is required to define exactly the process of particle formation and particle agglomeration during copper casse formation.

The electron microscope photographs showed little variation in particle size at different stages of the reaction. The particles appeared in various

TABLE 1

Composition of wines used

	Wine Type	Total acid as tartaric g./100 ml.	Volatile acid as acetic g./100 ml.	Ethyl alcohol vol. per cent	[1]Extract g./100 ml.	Reducing sugars g./100 ml.	Phosphate mg. PO_4/100 ml.	Tannin mg./100 ml.	Sulfur dioxide mg./liter		pH	Copper mg./liter	Iron mg./liter	Protein 10% TCA ppt. mg./liter
									Free	Total				
1	Sauterne	0.600	0.048	12.4	....	0.810	49.3	34.0	148	333	3.30	0.5	4.6	15.4
2	French Colombard	1.05	0.054	10.5	3.1	0.10	33.6	19.0	1.5	16.5	3.40	0.10	2.0	30.6
3	Semillon Blanc	0.86	0.036	13.1	2.7	0.09	9.12	15.0	1.1	9.0	3.12	0.23	5.2	98.0
4	Clairette Blanche	0.77	0.043	11.3	2.6	.....	31.7	15.75	1.0	9.2	3.40	0.0	2.1	111.0
5	French Colombard	0.83	0.033	14.0	2.5	0.07	21.9	19.0	1.1	7.5	3.25	0.0	5.3	.....

[1] Total soluble solids content determined by evaporation in drying oven (2).

TABLE 2

Copper and turbidity changes during formation and solution of copper casse

Sample	Time, days	Average turbidity	Copper content of wine supernatant mg. per 100 ml.	Copper content of precipitate[1] mg.
		Precipitate formation		
1	0	0	0.53	0.0
2	0.8	11	0.52	0.02
3	3	27	0.46	0.017
4	4	37	0.35	0.173
5	5	45	0.26	0.238
6	9	55	0.23	0.279
7	18	64	0.12	0.31
		Precipitate solution		
8	0	64	0.12	0.31
9	1	28	0.365	0.11
10	3	20	0.41	0.066
11	8	14	0.42	0.05
12	10	13	0.44	0.03

[1] Total copper content in precipitate formed in 100 ml. of wine.

states of agglomeration varying from single particles to many clumps at each interval. Since the electron microscope technique requires a thorough washing of the suspended particles, the removal of salts which serve as peptizing agents may have caused undue agglomeration, so that the true state of dispersion of the precipitate could not be observed. The particle size for the precipitate formed in wine 1 was estimated to be 15-40 mμ in diameter, as compared to 50-80 mμ for that in wine 3.

Hydrogen peroxide was added to a sample of the isolated precipitate and this was observed in the electron microscope after partial solution. A reduction in particle size was observed, but, more significant, a marked decrease in particle density as compared with the untreated sample occurred. The density of copper is much greater than any of the other constituents of the precipitate, namely: carbon, hydrogen, oxygen, nitrogen, and sulfur. This suggests that copper is readily dissolved out of the particles by this treatment.

The effect of bottle headspace on turbidity intensity. A considerable fluctuation in turbidity between identical bottles was observed in the previous experiment. The following experiment was designed to determine its cause and to study the limiting components involved in the copper casse reaction.

Procedure. Two series of jars containing wine 1 were prepared, and stored in an incubator at 45° C. One contained wine at 5 mg./liter of copper and the other contained 0.5 mg./liter copper. The former series was varied in headspace by introducing wine to each jar in 10-ml. increments. Thus there were jars, in duplicate, each containing 100, 90, 80, and 70 ml. of wine. None of these were nitrogen swept. For comparison, jars containing 100 ml. of wine were prepared and swept with nitrogen. The jars of wine containing 0.5 mg./liter copper were not nitrogen swept. At the end of 29 days 0.45 mg. copper were added under nitrogen in the cupric and cuprous states to different bottles containing wine at an initial copper content of 0.05 mg./100 ml. At the end of 34 days, this was re-

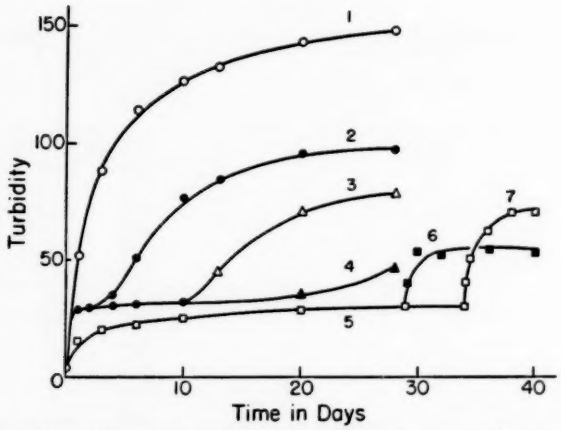


Figure 1. Effect of headspace and time of addition of copper and sulfur dioxide on copper casse formation in wine #1 at 45° C.

(1) 5 p.p.m. Cu, 100 ml. wine under nitrogen
(2) 5 p.p.m. Cu, 100 ml. wine under air
(3) 5 p.p.m. Cu, 90 ml. wine under air
(4) 5 p.p.m. Cu, 80 ml. wine under air
(5) 0.5 p.p.m. Cu, 100 ml. wine under air
(6) 4.5 p.p.m. cupric or cuprous added under nitrogen
(7) 4.5 p.p.m. cupric or cuprous and 200 p.p.m. SO_2 added under nitrogen

peated with the further addition of 200 mg. of sulfurous acid. Turbidity measurements were made periodically.

The data obtained, plotted in Figure 1, indicate that oxygen depletes one or more constituents in the wine and limits the extent of copper casse formation; that the addition of sulfurous acid restores only a part of the system, and that it definitely plays a role in copper casse formation; and finally, that the rate-limiting step in the reaction is independent of the form in which copper is added.

If the reaction as proposed by Ribéreau-Gayon requires that the copper be reduced to the cuprous state, then this step must be fast (at least when the wine is in the reduced state as it was at the end of 30 days), and not rate-determining since addition of both forms resulted in the same change in turbidity. This is true also for wines to which both forms of copper have been added at zero storage time, as shown by other experiments.

Although the constituents which contribute to the cloud formation and which react with oxygen are unknown, there are two possible explanations of the effect observed. Either the unknown reducing component, RH, may have been largely oxidized by the excess oxygen present, or the precipitate formed with 0.5 mg./liter copper may have contained a large proportion of the colloids necessary to the reaction.

The effect of reducing agents. A variety of reducing agents was added to wine 3 containing copper at a concentration of 5 mg./liter with and without added sulfur dioxide. These samples were nitrogen swept and stored at 35° C.

None of the phenolic type compounds tested had any effect on the rate of turbidity formation or final turbidity intensity. These were added at a concentration of 200 mg./liter and included pyrogallol, catchol, guaiacol, hydroquinone, dopa, and metol. Hydroxylamine hydrochloride had no effect. The sulfhydryl-containing compounds (cysteine and thioglycollic acid) gave flocculant precipitates when added; however, there was no change in turbidity with time. Sodium sulfide gave an immediate precipitate, part of which appeared to be free sulfur. Ascorbic acid, 200 mg./liter, caused a more rapid precipitation than the sulfur dioxide at 300 mg./liter.

Effect of temperature. Wines 1, 2, 3, and 4 were incubated at 35° C. and 45° C. for 20 days after adjusting the copper concentration to 5 mg./liter and the sulfur dioxide to 300 mg./liter. Copper was added in both the cupric and cuprous forms.

Assuming the reaction to be essentially complete when the turbidity ceases to increase, the half-time of reaction for the samples containing 300 mg./liter sulfur dioxide is given in Table 3.

TABLE 3

Effect of temperature on rate of increase in turbidity

Wine	Final turbidity intensity	Half-time of reaction in days	Protein content mg./liter
	at 35° C.		
1	125	7	15.4
2	40	1.5	30.6
3	310	0.5	98.0
4	330	0.6	111.0
	at 45° C.		
1	165	2.2	15.4
2	80	1.2	30.6
3	575	0.4	98.0
4	610	0.5	111.0

The values for the half-time of the reaction or reactions showed a small apparent temperature coefficient for the reaction rate. (These wines showed little or no clouding in the absence of copper; and the oxidation state of the added copper had no effect on the rate). There was an inverse relation between protein content and reaction half-time. Final turbidity intensity, on the other hand, increased with both temperature and protein concentration. The small apparent temperature coefficient and the variation of rate with the protein content indicated a low energy of activation and strongly suggests an adsorption process as a possible rate-limiting step in the reaction. However, at low protein levels, as in wine 1, there was a higher apparent temperature coefficient and thus a higher energy of activation such as is required for chemical reactions.

Effect of various factors on the rate of formation and intensity of turbidity. The effect of varying the major constituents in wine was studied in relation to changes in redox potential and copper casse formation *(16)*. However, quantitative turbidity data was not obtained at that time. In the following experiment these factors were varied over a wide range.

Procedure. Two wines were adjusted to 5 mg./liter copper, one was wine 1, containing 275 mg./liter total sulfur dioxide of which 48 mg./liter were free. The other was a composite of wines 1, 2, and 3 mixed in percentages of 24, 70, and 69, respectively, giving a final mixture containing 75 mg./liter total sulfur dioxide of which 12 mg./liter were free; 73.8 mg./liter protein; 0.4 mg./liter copper, and having a pH of 3.20.

The wines were varied as follows: pH from 2.5 to 5.0 in half pH unit increments; ethyl alcohol at 20 and 25%; invert sugar at 1 and 5%; tannic acid and monosodium-dihydrogen phosphate at 50 and 100 mg./100 ml.;[a] iron at 10 and 20 mg./liter; copper at 0.4, 3.0, 5.0, 15.0, and 50 mg./liter. The total sulfur dioxide content was in wine 1 adjusted from 275, 350, 500, 750, and 1,000 mg./liter, of which 48, 128, 278, 525, and 778 mg./liter, respectively, were free. The range of sulfur dioxide content in the composite wine was 75, 175, 325, 425, 525, 775, and 1,025 mg./liter of which 10, 110, 260, 360, 460, 710, and 960, respectively, were free.

Of the constituents that were varied, pH and sulfur dioxide content had the greatest effect on turbidity. The effect of the other factors varied is summarized in Table 4 in which the initial rate of increase in turbidity and the initial and maximum turbidity, observed over a storage period of 14 days for wine 1 and 19 days for wine 2, are given. In comparison with the control wine containing 5 mg./liter of copper and a sulfur dioxide level of 275 mg./liter for wine 1 and 325 for wine 2 (the mixed wine), increasing the alcohol content increased the initial rate of increase in turbidity and the maximum turbidity attained, particularly in wine 2. The addition of invert sugar and also of glucose and glycine decreased the rate of increase in turbidity and the maximum turbidity attained. Tannic acid also increased both rate and intensity of turbidity, but this may have been due to precipitation of proteins present. The phosphate had little effect on both rate of increase and intensity of turbidity. Increasing the iron content caused an immediate increase in turbidity owing to formation of iron casse under the initial oxidizing conditions present and this decreased as the wine became reducing; but it is doubtful whether it had an effect on copper casse. Wide variation in copper content had little if any effect on rate of increase in turbidity or maximum turbidity.

The effect of varying pH of the wine is shown in detail in Table 5. Decreasing pH to 2.5 appreciably increased rate of increase in turbidity, from 4.5 to 6 units per day in the control for wine 1, and from about 50 to 60 in wine 2. Increasing the pH from the initial level caused a progressive decrease in rate of increase in turbidity and also a decrease in maximum turbidity intensity. At pH 4.0 and above in wine 1 and pH 4.5 and above in wine 2 turbidity did not occur on storage.

Variation in sulfur dioxide content had the most noticeable effect on turbidity. The change in turbidity values for wines 1 and 2 with change in sulfur dioxide content are given in Table 6.

The data in Table 6 show a definite relation between turbidity and sulfur dioxide content. The initial slopes of the plots turbidity versus time at the various sulfur dioxide concentrations were estimated and when the

[a] The wines were adjusted to original pH after addition of phosphate.

TABLE 4

Effect of additives on initial rate of increase in turbidity and on intensity of turbidity

Factor varied	Wine 1			Wine 2		
	Initial rate[2]	Turbidity		Initial rate	Turbidity	
		Initial	Maximum		Initial	Maximum
Control	4.5	8	38	50	23	260
Alcohol						
20%	7.0	6	39	176	12	375
25%	27.0	6	51	235	13	370
Invert sugar						
1%	2.0	7	23	56	13	170
5%	1.0	7	17	41	13	140
Tannic acid						
50 mg./100 ml.	51	9	80	200	22	425
200 mg./100 ml.	64	24	147	300	47	433
Sodium phosphate						
50 mg./100 ml.	2	6	27	43	14	132
200 mg./100 ml.	1	7	21	60	14	250
Glucose: glycine						
1%	0	7	9	....	....	....
5%	0	8	6	....	....	....
Iron						
4.6[1] (1); 5.2[1] (2)	2	7	18	60	14	250
10 p.p.m.	3	9	28	50	29	165
20 p.p.m.	−150	197	70	15	125	250
Copper						
0.5[1] (1); 0.4[1] (2)	0	12	20	40	13	172
3.0 p.p.m.	2	7	17	60	14	250
5.0 p.p.m.	0	11	25	....	....	....
15.0 p.p.m.	1.5	7	17	73	15	200
50.0 p.p.m.	....	....	....	90	15	240

[1] Initial level in wine 1 and wine 2, respectively.
[2] Expressed as increase in turbidity reading per day.

log of the initial slopes was plotted against the log of the free sulfur dioxide concentration, a straight line resulted with a slope close to one, as shown in Figures 2 and 3 for wines 1 and 2, respectively. These results suggest the reaction was first order with respect to free sulfur dioxide.

The relation between rate of turbidity change and free sulfur dioxide concentration is only inferred since neither the actual change in reactant or product concentration were measured. If it is assumed that the rate of change of intensity of scattered light (dT) under the experimental conditions used, is directly proportional to the rate of formation of the unknown reaction product or

$$\frac{dT}{dt} = k \frac{d\ (product)}{dt}$$

then it follows that since

$$\frac{dT}{dt} = k\ (free\ SO_2)$$

that

$$\frac{d\ (product)}{dt} = k\ (free\ SO_2)$$

TABLE 5

Effect of pH on increase in turbidity with time of storage

Wine	pH	Days stored										
		0	1	2	3	4	5	6	8	9	11	12
1	3.0	8	12	17	21	26		27		31	29	
	2.5	8	16	20	26	32		38		38	36	
	3.3[1]	7	5	9	12	14		17		18	17	
	4.0	7	5	5	6	5		5		5	4	
	4.5	6	5	5	4	4		4		4	3	
	5.0	5	4	3	4	3		4		4	3	
2	2.9	23	70	130	174	....	198	....	222	....	....	235
	2.5	27	54	111	170	....	207	....	264	....	....	308
	3.2[1]	14	74	112	138	....	157	....	184	....	....	215
	4.0	12	12	16	19		22		24			26
	4.5	11	9	10	9		10		9			9
	5.0	8	8	8	8		8		7			7

[1] Initial pH of wine before adjustment.

This assumption is valid if the light-scattering intensity is due only or primarily to new particle formation of approximately the same size and if no particle aggregation occurs during the initial period of the reaction. Light-scattering intensity could then be used as a measure of the rate of product formation during this period.

These assumptions have not been substantiated by direct evidence. In order to establish a definite relation between turbidity and product formation, it would be necessary to determine the actual changes in product concentration in relation to turbidity change with time.

Model synthetic systems. In order to study the nature of the reaction between sulfur dioxide and protein and to determine whether a reaction occurs between copper and sulfur dioxide and protein, and if a reaction

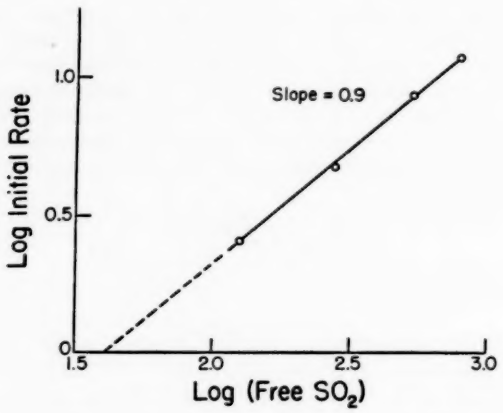


Figure 2. Relation of initial rate of turbidity formation to free sulfur dioxide content in mg./liter in wine #1 at 35° C.

TABLE 6

Effect of sulfur dioxide content on rate of increase in turbidity

Wine	Sulfur dioxide content (mg./liter)		Turbidity ($H_2O = 0$) Days stored											
	Total	Free	0	1	2	3	4	5	6	8	9	11	14	17
1	275	48	7	5	9	12	14		17		18	17	17	19
	350	128	7	11	11	14	19		23		25	24	28	26
	500	278	7	13	16	22	29		38		50	61	90	100
	750	525	6	16	27	35	46		62		74	85	108	119
	1000	778	7	20	34	43	59		80		97	112	138	144
2	75	10	12	39	38	38		42		44				
	175	110	13	52	57	64		74		90				
	325	260	14	74	112	138		157		184				
	425	360	14	99	140	175		206		277				
	525	460	14	124	170	210		262		326				
	775	710	15	171	231	393		343		416				
	1025	960	15	187	253	313		368		462				

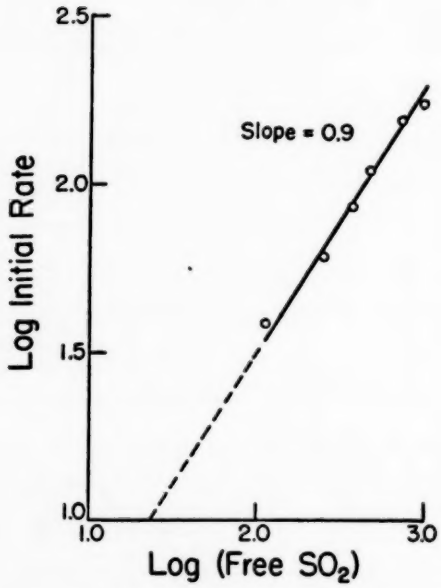


Figure 3. Relation of initial rate of turbidity formation to free sulfur dioxide content in mg./liter in wine #2 at 35° C.

occurs between copper and sulfur dioxide in the absence of sunlight, reducing agents or surfaces other than glass, the following samples were prepared.

Procedure. A solution of 0.75% tartrate at pH 3.5 adjusted to 5 mg./liter copper and 300 mg./liter sulfur dioxide was prepared. Three sets of samples were prepared in pyrex and soft-glass bottles as follows: (1) deaerated, glass-stoppered; (2) not deaerated, glass-stoppered; and (3) not deaerated, rubber-stoppered. The samples were stored at 35° C. in the dark. After deaerating and sweeping with nitrogen a fourth series was prepared by the addition of various sources of sulfur and nitrogen to two-liter bottles. Insulin was added to one at a concentration of 20 mg./liter; lactalbumin hydrolysate to another at a concentration of 300 mg./liter; cystine at 100 mg./liter to another; 1.0 g./liter of ascorbic acid and 100 mg./liter cystine to another (no sulfur dioxide was added to this); and in a fifth bottle, as control, nothing was added. The flasks were stored in a 35° C. incubator. Samples were withdrawn periodically under nitrogen and analyzed for copper, sulfur dioxide, sulfate, and turbidity.

After storage for 2½ months the first 3 series of samples showed no change. At the end of 4½ months a haze was visible in sample (1) pyrex glass, and gave a turbidity reading of 40. (The other samples were not stored this long). On addition of H_2O_2, the turbidity immediately dropped to 12 and within an hour to 5. The final sulfur dioxide concentration was 170 mg./liter.

TABLE 7

Changes in turbidity, copper, sulfur dioxide, and sulfate content in model systems

Additive	Copper content, p.p.m.			Sulfur dioxide mg./liter			Sulfate mg./liter			Turbidity		
	3 hr.	1 day	4 days	3 hr.	1 day	4 days	3 hr.	1 day	4 days	3 hr.	1 day	4 days
Control	5.2	5.2	5.2	279	269	248	21	52	54	0	0	0
Insulin	5.2	5.2	5.2	282	269	250	21	52	50	17	109	109
Lactalbumin hydrolysate	5.2	5.2	5.2	269	267	250	37	49	49	0	0	0
Cystine	5.2	0.3	0.2	269	251	197	29	33	49	2	370	370
Cystine and ascorbic acid	3.4	1.4	0.7	0	0	0	0	0	0	350	500	500

In the fourth series, as shown in Table 7, the control sample did not become turbid after 3 days and even after storage for over 2 months remained clear. The sample with insulin added was cloudy in 3 hours and reached its maximum turbidity in one day. No appreciable changes in copper content occurred, however, and the changes in sulfur dioxide and sulfate content were similar to those in the control. The sample with lactalbumin hydrolysate, added as a source of polypeptides and lower molecular weight peptides, did not become cloudy and was similar to the control in sulfur dioxide and sulfate content. In other experiments the addition of lactalbumin hydrolysate in presence of sulfur dioxide both to tartrate solutions, with and without alcohol, or to wines did not cause appreciable change in turbidity or in copper content. This was also true of albumin. Casein or gelatin could not be used in wines containing appreciable tannin owing to formation of insoluble tannates. In the sample containing cystine, haze formation occurred and this reached a maximum turbidity after one day of storage and this remained constant. Paralleling this increase in turbidity was the decrease in copper content in the solution phase and an appreciable decrease in sulfur dioxide content which was greater than in the control, although the accumulation of sulfate was less. In the sample in which ascorbic acid served as the reductant instead of sulfur dioxide, the turbidity increased more rapidly and reached a higher level and copper began to precipitate sooner but did not reach as low a level as in the cystine-sulfur dioxide system. Clouding and precipitation of cystine in the presence of copper did not occur in the absence of a reducing agent.

The precipitates obtained from the cystine samples were isolated and analyzed with the results shown in Table 8 in comparison with the composition of a theoretically possible copper cysteinate. With glycine, Dobbie *et al. (6)* reported that one proton was given up for each molecule of amino acid complexed by copper and although similar data for cysteine is not available it was assumed to be true for it also. In view of the fact that the composition of the precipitate was essentially the same whether sulfur dioxide or ascorbic acid served as the reductant, it would appear that sulfur dioxide was not directly involved and that reduction of cystine to cysteine occurred in the process. With insulin, which contains 12% cystine *(19)*, no reaction with copper occurred. The turbidity which formed in its presence may have been due to denaturation with subsequent flocculation. Apparently, it was denatured faster than copper could complex with it or the sulfhydryl groups freed during denaturation were not available for complexing.

TABLE 8

Elemental analyses of precipitates obtained by the reduction of cystine in the presence of copper, in % by weight

Sample	Cu	S	N	C	H	Residue on ignition
Cu-cystine-sulfur dioxide	30.4	14.95	5.8	18.1	3.64	50.0
Cu-cystine-ascorbic acid	32.3	13.91	5.9	17.35	3.51	53.1
Theoretical Cu-cysteinate	34.6	17.4	7.6	19.6	3.3	

Although these data are not sufficient to prove the conversion of cystine to cysteine in the system investigated or that denaturation of protein was involved they are suggestive of this.

Mirsky and Anson *(22, 23)* studied the changes in the sulfur-containing amino acids which occur on denaturation, and showed that all of the -SH and -S-S- groups of proteins are accessible and reactive in denatured or unhydrolyzed proteins. This was shown to be the case when proteins were denatured by heat, TCA, urea, ultra-violet light, and shaking in air. The native proteins have either no reactive -SH and -S-S- groups or only a fraction of those which are detectable in denatured proteins.

The appearance of sulfhydryl groups is characteristic of the denaturation process resulting in a decreased protein stability and solubility. In wine, containing free sulfur dioxide a similar reaction may occur, that is, the sulfur dioxide may act as a denaturing agent. After denaturation, the protein would be flocculated by the copper complex, the rate of which would depend on the protein concentration and its availability for reaction with sulfur dioxide.

The nature of the effect of sulfur dioxide on proteins was not investigated by us, but there is evidence in the literature that disulfide bonds in proteins undergo a reaction wtih sulfiite leading to the formation of free sulfhydryl groups. This reaction, in which one mole of -S-S- yields one mole of -SH was termed sulfitolysis by Kolthoff and Lingane *(18)*

$$RSSR + SO_3^{--} \leftrightarrows RS^- + RSSO_3^-$$

and used as the basis of amperometric determination of disulfides in intact protein by Carter *(5)*.

STUDIES ON THE PRECIPITATE

The precipitates from wines 1 through 4 were isolated by centrifugation. They were washed several times with cold distilled water and dried over calcium chloride in a vacuum desiccator. The precipitates were analyzed; X-ray diffraction pictures were taken and their electrophoretic mobility was studied.

Chemical analysis of the copper precipitates. The chemical compositions of the precipitates isolated are shown in Table 9. The initial and final

TABLE 9

Chemical analysis of copper precipitates

Wine	Maximum turbidity	TCA protein (mg./liter)		% Cu	% S	Cu/S mole ratio	% N	% protein[1]	Weight of precipitates isolated (mg./liter)
		Initial	Final						
1	173	15.4	7.0	14.0	7.85	0.906	9.3	58.2	20
2	77	30.6	20.1	2.88	3.89	0.279	10.7	67	18
3	385	98	54	3.04	4.58	0.337	17.8	111	78
4	350	111	71	1.07	2.85	0.191	10.0	62.5	64

[1] On the usual assumption that proteins contain an average of 16% nitrogen.

protein contents were the values obtained in the wine before and after copper casse formation.

The per cents carbon and hydrogen and residue on ignition of precipitate No. 1, were 37.39, 5.85, and 19.5, respectively. The analyses show the presence of copper and sulfur in all cases but not in constant ratio to the amount of nitrogen present.

High molecular weight colloids have been implicated in the formation and flocculation of the insoluble copper compounds responsible for copper casse. Although Dubaquié *(8)* suggested that pectic substances were involved and stated that precipitation of cuprous and ferrous compounds by the proteins of wine was inhibited by sulfur dioxide, the bulk of the evidence indicates that polypeptides or proteins are involved *(16, 26)*. Nitrogenous compounds have been reported to be present in all cases but the nature of these compounds, and their function, is not known. It is possible that they may occur in both the primary micelle formation and in the secondary aggregates formed. The term "protein" is used here in the general rather than particular sense.

The amount of precipitate isolated indicates that high protein wines give more copious clouding than do low protein wines, but the depletion in protein content is not directly related to its relative percentage in the precipitate. The high protein figure for precipitate No. 3 indicates nitrogen is present in an organic compound having less than the usual empirical percentage of 16.0 used in converting nitrogen to protein. The sulfur figures represent total sulfur and do not differentiate between that attached to copper, that present in the protein, or that which may be adsorbed on the precipitate surface as sulfur dioxide or in other forms. Thus, it was impossible to establish a correlation between copper and sulfur as indicated by the randomness of the Cu/S mole ratios obtained for the several precipitates.

The precipitates were dissolved in 0.1 N sodium hydroxide with gentle heating. The resulting solutions were brown to black in color. On acidifying again with 0.1 N hydrochloric acid, the precipitates flocculated out of solution. The samples were centrifuged and the precipitate and supernatant were analyzed for copper. Practically all the copper was in the supernatant. Using indicator paper, it was observed that the flocculation occurred in all the precipitates between pH 3.5 and 4.0.

Electrophoresis studies. The electrophoresis apparatus was a commercial model with a full wave rectifier circuit with a voltage range from zero to 750 volts at 200 milliamperes. The voltage regulation was approximately 1% and the plastic paper supporting area between the electrodes was water cooled.

No 3 Whatman paper strips 25 inches long were wet and blotted with 0.1 M phthalate buffer at pH 4.0 and 6.0; at pH 8.0, 9.0, and 10.0 a 0.1 M borate buffer was used. The solutions obtained by dissolving the precipitate in 0.1 N sodium hydroxide were applied as spots at the center of the paper and a voltage of 300 at from one to 10 milliamperes, depending on pH, was applied for 3 to 6 hours.

Copper was detected by the sodium dithiodiethylcarbamate method as used in the colorimetric determination.. A method utilized successfully by

Durrum *(10, 11)* was employed for protein detection. The paper strips were first immersed for 5 minutes in a saturated mercuric chloride, ethyl alcohol solution containing 0.1% brom-phenol blue dye. It was then washed with running water for 10 minutes. The excess dye was then washed out, leaving the paper colorless except where the protein had been fixed to the paper.

Approximately the same mobility of copper and protein was found in all solutions. At pH 4.0, there was practically no mobility. There was mobility toward the cathode at pH 7.0 and this was more pronounced at pH 8.0. At pH 9.0 and 10.0 a protein spot moved toward the anode while the copper continued to move toward the cathode. Thus, between pH 8.0 and 9.0 a separation of copper and protein occurred. This indicated a change in protein from positive below pH 8.0 to negative above pH 9.0.

At pH 11 the copper was detected as a streak, usually with the greatest concentration at the spot head and diffusing back toward the center where the spot was applied, indicating a wide range in particle size. The protein, on the other hand, appeared as a well-defined spot at pH 9.0 and 10.0.

A positive protein reaction was observed at all pH values, in spots migrating toward the cathode. This was true even at pH 9 and 10. At these pH values, however, a protein spot moving toward the anode was also evident. This may have been caused by either the dye adsorption process not being specific for protein and giving a reaction with any colloid which is fixed onto the paper; or by two proteins being involved, one of which separates above pH 9.0 and moves toward the anode and the other which may or may not be attached to the copper sulfide, which moves toward the cathode. It remains to be demonstrated whether nitrogenous or other material moves with the copper above pH 9.0.

X-ray diffraction studies. Chemical methods were inadequate to establish the nature of the sulfur compound in the precipitates. It was necessary to resort to X-ray diffraction studies in order to demonstrate whether sulfur was attached to copper or was a part of the protein.

Procedure. The precipitates were mounted either in a fine-walled capillary or on a thin glass thread using collodion as the adhesive. It was found the latter technique gave better results.

The samples were exposed to copper $K\alpha_1$, $\lambda = 1.5405$ radiation for a period of about two hours after which the film was developed, dried, and the resulting lines measured. The line-measuring device could be read with an accuracy of 0.01 centimeter so that the largest sources of errors in reading the lines were in centering the indicator on the lines, on the line optical density, and on the width of the lines.

After the lines were measured, their values (D) were converted to Ångström units by the formula $\tan 2\theta = L/R$, where R is the distance in centimeters from the target to the camera. The tangent was taken to give 2θ values which were converted to Ångström units *(24)*. A search was made in the ASTM Card File Index *(1)* for the lines of suspected copper compounds. The index lists lines in Ångström units, determined from powder diagrams, by relative intensity and the line with the greatest optical density is given as 100 and all other lines are related to it according to their relative optical densities. Since an optical density measuring device

TABLE 10

X-ray data of copper casse index lines in Ångströms

Known compound		Precipitate					
Cu_2S observed Å	CuS observed Å	1 Å	2 Å	3 Å	4 Å	5 Å	6 Å
							3.45 w
		3.41 s		3.40 s	3.40 s		
3.37 w	3.39	3.37 w	3.35 s				
	3.18 s	3.34 s				3.23 w	3.21 s
3.00 s	3.00 vs	3.08 w	3.03 s			3.06 w	
2.736 s	2.78 vs	2.84 s	2.74 s	2.81 s	2.81 s	2.87 s	2.86 s
	2.68 s					2.63 vw	
2.49 vs		2.41 s	2.45 w	2.44 s	2.44 s	2.46 s	2.44 s
						2.40 vw	
2.31 vs	2.28 vw		2.25 w				2.37 s
							2.32 w
2.13 s	2.07 vw	2.20 w	2.08 s	2.09 w	2.09 vw	2.08 w	2.19 w
							2.08 w
1.96 vw	2.03 vw	2.09 w					
		2.03 w					
1.85 s	1.87 vs						
1.70 w	1.72 w			1.73 vw	1.73 vw		
1.57 w	1.56 w						
1.50 w		1.51 w					

vs—very-strong
s—strong
w—weak
vw—very weak

Unidentified lines	
	4.10 w
3.85 w	3.89 w
	3.64 w

was not available the relative intensities obtained are listed qualitatively in Table 10 as: vs (very strong), s (strong), w (weak), and vw (very weak).

On comparing the unknown with known lines, it was evident that both cupric and cuprous sulfides were present. There was some reason for doubt, however, in that both sulfides have many lines in common. Two distinguishing features serve to identify the two compounds: (1) the relative line intensities; (2) at 2.49 Å a strong line exists in the cuprous sulfide pattern and not in the cupric sulfide pattern. Since the copper sulfides were extremely dilute because of the presence of protein, only the strong lines appeared (although weak in these patterns as compared with the pure compounds), while the weak lines did not appear, or only very faintly. In a complex mixture, relative intensity loses some value as an identifying characteristic since relative line intensities may be considerably modified by the presence of interfering and disorienting reflecting surfaces.

An attempt was made to resolve these difficulties by preparing known compounds of cupric and cuprous sulfide and diluting a mixture of the two by five times with a wine precipitate, high in protein and low in copper. These patterns were compared with lines in the ASTM Index and for the most part they agreed within 1% in line location and also in relative line intensities. There was one notable exception, the strong lines at 1.95 and 1.87 Å for cuprous sulfide (ASTM) were found to be 1.96 (vw) and 1.87 (s) for the prepared sample. Also the lines at 2.49 and 2.31 Å were observed as vs while in the ASTM they were listed as 2.54 (50) and

2.38 (90). These differences can be accounted for by differences in camera technique as well as by variations in crystalline structure.

It was decided to use the measured lines of cupric and cuprous sulfides as comparison standards. These are listed in Table 10, with the estimated relative line intensities. The precipitates obtained from two commercial wines were also tested (5 and 6 in Table 10). All the lines except three in No. 5 could be accounted for as cupric or cuprous sulfide lines.

The lines given the most weight were those at about 3.00, 2.80, 2.49, and 2.31 Å. The first two are strong for cupric sulfide and relatively weak for cuprous sulfide so that these were considered as cupric sulfide and not cuprous sulfide lines. The reverse is true for the last two lines which are strong for cuprous sulfide. On this basis it was concluded that both sulfides were present in the precipitates.

The diluted mixture of the two sulfides showed the marked manner in which the lines were reduced in intensity and demonstrated that weak lines do not appear when diluted in this manner. It is notable that the two very strong lines for cuprous sulfide at 2.49 and 2.31 Å showed a marked dimunition in intensities, relatively more so than did other lines.

The precipitate from wine 1 was dialyzed for 48 hours against distilled water after which the X-ray pattern was determined. There was no change in the pattern, which indicated the copper micelles in the precipitate are of colloidal size.

DISCUSSION

Turbidity. The data obtained on the effect of sulfur dioxide on rate of turbidity increase can be correlated with the observed effect of pH on tha sulfite-bisulfite-sulfurous acid equilibrium. As Vas and Ingram *(27)* reported for water solutions, at pH 4.0 the free sulfurous acid concentration is almost zero but increases to about 20% at pH 2.5. The increase in turbidity with decrease in pH can thus be explained partly in terms of the free sulfur dioxide concentration. Another effect of pH is also contributory. As an increase in hydrogen ion concentration occurs, the increase in total positive charge exerts a suppressive effect on the zeta-potential of the protein, making it more susceptible to flocculation by the adsorbed copper complex.

High concentrations of alcohol exert similar action. Here, however, the decreased protein stability is caused by a dehydration and consequent reduction of protein solubility. At the levels of alcohol used, a precipitate formed only in the presence of copper in the reduced wine. The inhibitory effect of high sugar concentration exerts the opposite effect, that is, protects the protein from being denatured.

It was observed that the rate was independent of the copper concentration above an unknown limiting amount necessary for the reaction. Copper, therefore, is not involved in the rate-determining step of the reaction. Its reduction and subsequent reaction with sulfur dioxide to form hydrogen sulfide must be fast with respect to the over-all rate. This was demonstrated by Ribéreau-Gayon who showed that by fining wines not susceptible to copper casse (low protein content) with isinglass after heating to 80° C. for one-half hour and allowing them to cool, all the copper was removed from solution. The coagulated isinglass gel was col-

ored red-brown and was darker the higher the copper concentration. Analysis showed the colored material to contain copper and sulfide. Thus, under the influence of heating, the copper was transformed from the cupric state, incapable of being eliminated by fining, to the state of copper sulfide in a clear colloidal solution capable of being flocculated and precipitated by fining.

The reaction was postulated to be first order with respect to free sulfur dioxide concentration. This was based on the assumption that the rate of light scattering was directly proportional to the rate of product formation and that light-scattering intensity change was the result of primarily new particle formation of approximately the same size with little or no particle aggregation during the initial period of the reaction.

A wide variety of reducing agents showed no effect on the reaction. The protein concentration was observed to affect markedly both the rate and final turbidity intensity. The dependence of rate on temperature was defined by the amount of protein present.

High temperatures resulted in a greater turbidity intensity; however, the rate appeared to be independent of temperature at high protein concentration but temperature dependent at low protein concentrations. The apparent temperature coefficient was very low in wines high in protein and increased with decreasing protein content to that characteristic of chemical reactions.

Precipitate studies. The precipitate in all cases contained copper, sulfur, and nitrogen. The amount of precipitate isolated was related to the amount of TCA-precipitable protein in the wine. The stoichiometric relationships between the various components of the precipitates were not constant and could not be defined.

X-ray diffraction studies demonstrated the presence of cuprous and cupric sulfides. The electrophoresis studies demonstrated a separation of copper and protein at pH 9 and above.

Proposed theory. The presence of sulfur, protein, and copper as the sulfides in the precipitates limits the speculative possibilities. However, several reactions indicated by the data may be proposed.

The existence of cuprous sulfide in the precipitates requires the reduction of cupric to cuprous ion. This may be accomplished in several ways. The reactions between cupric ion and sulfrous acid to give both cuprous and cupric sulfides are thermodynamically possible, the formation of cuprous sulfide being favored. Results which could be attributed to these reactions were obtained. These reactions, however, occurred at a slow rate. They may require a surface, such as that of the protein, on which the reaction can take place at a measurable rate.

Another reaction may occur, as previously suggested by Ribéreau-Gayon. This is probably a faster reaction involving an unknown reducing agent, RH:

(1) $Cu^{++} + RH \rightarrow Cu^{+} + R + H^{+}$

The source of sulfide ion may be either sulfur dioxide or protein. If it is the former, the reduction of sulfur dioxide may occur as Ribéreau-Gayon proposed:

(2) $6\,Cu^{+} + SO_2 + 6\,H^{+} \rightarrow 6\,Cu^{++} + H_2S + 2\,H_2O$

If it is the latter, a dismutation of the protein molecule following its denaturation would occur. Neither reaction has been demonstrated.

The resulting hydrogen sulfide reacts with copper in both states of oxidation giving their corresponding sulfides. These reactions must be fast in relation to other steps in the over-all process since the rate is independent of the copper concentration.

The rate and intensity of turbidity formation were shown to be dependent on both the free sulfur dioxide and protein concentrations. Besides serving as a possible source of sulfide ion, the sulfur dioxide may function as a denaturing agent in the presence of metal ion catalysts. In the presence of cupric ion, sulfur dioxide reduces the dithio linkage in cystine to produce the free sulfhydryl group of cysteine. It was shown that sulfur dioxide, in the presence of cupric ions, caused the flocculation of insulin presumably causing its denaturation through a reductive mechanism.

If colloidal copper sulfide *(12)* is added to wine without sulfur dioxide, no precipitate results. The rate of copper casse formation is considerably increased as the ethyl alcohol content is increased. This effect is not observed in reduced wines not containing copper so that the effect is not a dehydrating effect on the native protein but rather on the denatured protein.

The above considerations lead to reaction *(3)* between sulfurous acid and native protein to give sulfate and denatured protein:

$$(3)\quad \underset{\text{native protein}}{H_2SO_3 + RSSR} + H_2O \rightarrow SO_4^{=} + 2\,\underset{\text{denatured protein}}{RSH} + 2\,H^+$$

The rate dependence on protein suggests reaction (4):

$$(4)\quad RSH + \underset{\text{or CuS}}{Cu_2S} \rightarrow \underset{\text{or RSH-CuS}}{RSH\text{-}Cu_2S}$$

At high protein levels, the rate-determining step may be the rate of adsorption of the copper sulfides on the denatured protein and at low levels, it may be the rate of protein denaturation. Flocculation of adsorbed copper sulfide and denatured protein then results.

LITERATURE CITED

1. American Society for Testing Materials (ASTM). Card File Index, 2nd ed., 2nd supplement. (1950).
2. Association of Official Agricultural Chemists. *Methods of Analysis*, 7th ed., 1950. Washington, D. C.
3. Carles. Troubles cuivriques. Soc. sciences physiques et naturelles de Bordeaux 1908, 48-54.
4. Carles. Les vins blancs cuivres. *Ann. fals.* 11, 48-51 (1918).
5. Carter, J. R. Amperometric determination of disulfides in intact proteins. *Science*, 120, 895-896 (1954).
6. Dobbie, H., Kermack, W. O., and Lees, H. Complex formation between polypeptides and metals. *Biochem. J.*, 59, 240-246, 246-257, 257-264 (1955).
7. Draper, W., and Thomson, J. L. Cloudiness in wines. *Chemistry in Canada*, 7 (8) 35-58 (1955).

8. DUBAQUIÉ, J. L'anhydride sulfureux et la tenue des vins blancs en bouteilles. *Ann. fals.*, **18**, 418-422 (1925).
9. DUBAQUIÉ, J. Nouvelle contribution à l'étude des troubles por reduction dans les vins blancs. *Ann. fals.*, **23**, 295-301 (1930).
10. DURRUM, E. L. A microelectrophoretic and microionophoretic technique. *J. Am. Chem. Soc.*, **72**, 2943-48 (1950).
11. DURRUM, E. L. Paper ionophoresis. *Science*, **113**, 66-68 (1951).
12. HAUSER, E. A., AND LYNN, J. E. *Experiments in Colloid Chemistry.* 1940, McGraw-Hill, New York, N. Y.
13. HOCH, F. L., AND VALLEE, B. L. Gravimetric estimation of protein precipitated by trichloroacetic acid. *Anal. Chem.*, **25**, 317-320 (1953).
14. JOSLYN, M. A., AND LUKTON, A. Prevention of copper and iron turbidities in wine. *Hilgardia*, **22**, 451-533. Calif. Agr. Expt. Sta. Jour., U. of Calif., Berkeley, Calif. (1953).
15. JOSLYN, M. A., AND LUKTON, A. On the mechanism of copper casse. Technical Advisory Committee, Wine Institute *Mimeo. Report*, Aug. 5. 1955,
16. JOSLYN, M. A., AND LUKTON, A. Mechanism of copper casse formation in white table wine. I. Relation of changes in redox potential to copper casse. *Food Research* **21**, 384-396 (1956).
17. KIELHÖFER, E. Die Eiweisstrübung des Weines. IV. Über die Beschaffenheit des Trübes und die Verminderung des N-Gehaltes des Weines durch die Trubabscherdung. *Z. Lebens.-Untersuch u. Forsch.* **92**, 1-9 (1951).
18. KOLTHOFF, I. M., AND LINGANE, J. J. *Polarography.* 2nd ed., 1952, Vol. 2: 944-45. Interscience Publishers, New York, N. Y.
19. LENS, J., AND NEUTCHINGS, J. The reduction of insulin. *Biochim. et Biophys. Acta*, **4**, 501-8 (1950).
20. LHERME, AND D'ESTIVAUX, J. BAILLOT. Le louchissement des vins blancs en bouteilles. Congrés intern vigne vin (Bordeaux) 1929: 107-113; *Chimie et Industrie*, **23**, 1510 (1930).
21. LUKTON, A. Factors affecting the formation of copper casse in white wines. (Ph.D. Thesis, Univ. of Calif., Berkeley, June, 1954.)
22. MIRSKY, A. E., AND ANSON, M. L. Sulfhydryl and disulfide groups of proteins. I. Method of estimation. *J. Gen. Physiol.*, **18**, 307-23 (1935).
23. MIRSKY, A. E., AND ANSON, M. L. Sulfhydryl and disulfide groups of proteins. *J. Gen. Physiol.*, **19**, 427-59 (1936).
24. NATIONAL BUREAU OF STANDARDS, AMS 10. *Tables for Conversion of X-ray Diffraction Angles to Interplanar Spacing.* U. S. Government Printing Office, Washington, D. C. (1950).
25. RENTSCHLER, H., AND TANNER, H. Über die Kupfersulfit-Trübung von Weissweinen und Süssmosten. *Schweiz. Z. Obst. Weinbau*, **60** (16) 298-301 (1951).
26. RIBÉREAU-GAYON. "Contribution à l'Etude des Oxydations et Réductions dans les Vins. 2nd ed., 1933, Delmas, Bordeaux, France.
27. VAS, K., AND INGRAM, M. Preservation of fruit juices with less sulfur dioxide. Food Manuf., **24**, 414-416 (1949).

AMINO ACID COMPOSITION OF SOME INDIAN VEGETABLES AS DETERMINED BY PAPER CHROMATOGRAPHY

S. GHOSH MAJUMDER, RABINDRA NATH DUTTA, AND N. C. GANGULI
Department of Applied Chemistry, University College of Science and Technology, Calcutta, India

(Manuscript received February 20, 1956)

Information regarding the amino acid composition of common Indian vegetables of dietary interest is very meager. Because of its bearing on nutrition, it is of great value to have a complete account of the amino acid composition of Indian vegetables.

This paper reports on the amino acid composition of vegetables commonly available in the daily market. Quantitative values and the detection of each amino acid were accomplished with the aid of both unidimensional and two-dimensional paper chromatography.

MATERIALS AND METHODS

Materials. Twenty varieties of common Indian vegetables were collected from three different local markets. Five g. of each sample was finely chopped and used as the test material.

Preparation of sample. Five g. of each sample was placed in each test tube and hydrolysed with 5 ml. of 5.7N HCl for 24 hrs. at 105° C. according to the method of Holmes *(7)*. After hydrolysis, excess HCl was removed by repeated evaporation, as is usually done in preparing samples of protein hydrolysate for paper chromatographic analysis *(2)*. Finally, the hydrolysate was made up to a volume of 4 ml. with 10% isopropyl alcohol. A few drops of toluene were added to each tube in order to prevent bacterial contamination. These hydrolysate were then used for analysing the respective amino acids (except tryptophan). The solutions were kept in refrigerator when not in use.

Quantitative estimation of amino acids by paper chromatography. For the estimation of amino acids two sets of experiments were performed: one with a 10-μl. solution and the other with a 20-μl. solution of each hydrolysate. It has been observed that an inadequate application of the sample sometimes occurs in paper chromatographic analysis and results in incomplete information regarding the number of amino acids actually present. Amino acids which are present in minute quantity may not appear. For this reason two different proportions of the sample solution were applied on separate sheets of paper for analysis.

The methods applied by Block and Weiss *(3)* in analysing bovine whey proteins and by Bagchi, Ganguli, and Roy *(1)* in analysing some pulse samples are followed here for the complete evaluation of the amino acid composition of the vegetable samples.

Whatman No. 1 filter paper sheets, 52 × 46 cm., were used for two-dimensional chromatography, with (1) phenol-water and (2) *n*-butanol-acetic acid-water (4:1:1) as solvents. Cystine, lysine, threonine, alanine, tyrosine, methionine, valine, leucines, phenylalanine, and histidine were determined on this two-dimensional chromatogram. After development the paper was air-dried and sprayed with 0.5% ninhydrin solution and heated in the electrically heated air oven for a period of 30 min. at 75° C. The coloured spots, after proper identification, were eluted with a 75% alcoholic solution containing 5 mg. of $CuSO_4$, $5H_2O$ per 100 ml. of solution *(6)*. Each spot was extracted with 5 ml. of this solution for 10 to 15 min., by which time all the coloured matter will come out of the filter paper. The colour intensities of these solutions were then measured in a Klett-Summerson photoelectric colorimeter using 550 mμ filter. Complete evaluation of the amount was then made from standard curves of each amino acid, prepared previously, using the same method of chromatography and elution.

Aspartic acid, glutamic acid, serine, and glycine were separated on one-dimensional chromatograms with 72% aqueous phenol in the presence of HCN and 0.3% NH_3 *(3)*. Cystine, lysine, histidine, arginine, alanine, tyrosine, and phenylalanine were also separated using n-butanol-acetic acid *(4)*. Histidine was estimated from the butanol-acetic acid chromatogram with Pauly reagent *(4)* and proline with isatin on a phenol-water chromatogram using the "area of spot" method *(5)*.

Protein percentage of each sample was determined by the usual Kjeldahl method.

TABLE 1

Local and botanical names of the vegetables analysed for their amino acid composition

Name of the foodstuff	Local name	Botanical name	Protein	Moisture
			%	%
Spinach	Palang sag	*Spinacia oleracea*	2.3	91.5
Carrot	Gajar	*Daucus carota*	0.95	86.0
Beet root	Beet	*Beta vulgaris*	1.6	83.5
Tomato (green)	Kancha Bilati Begun	*Lycoperiscum esculentum*	1.8	92.8
Tomato (ripe)	Paka Bilati Begun	*Lycoperiscum esculentum*	0.8	94.2
Cauliflower	Phoolkopee	*Brassica oleracea botnytes*	3.5	89.0
Cabbage	Bhanda kopee	*Brassica oleracea capitata*	2.0	90.2
Peas	Matar soti	*Pisum sativum*	6.5	72.0
Pumpkin	Kumra	*Cucurbita maxima*	1.5	92.5
Pumpkin stalks	Kumra sag	*Cucurbita maxima*	2.0	90.2
Neem tender	Kachi neem pata	*Azadirachta indica*	3.8	62.8
Onion stalks	Payaj kauli	*Allium cepa*	0.8	80.5
Onion	Payaj	*Allium cepa*	0.6	86.3
Ridge gourd	Jhinga	*Luffa acutangula*	0.6	95.0
Radish	Mula	*Raphanus sativus*	0.8	94.2
Radish stalks	Mula sag	*Raphanus sativus*	1.2	91.3
Sweet potato	Ranga alu	*Ipomeoa batatas*	1.2	68.2
Amaranth tender	Banopata nate	*Amaranthus gangeticus*	5.0	85.6
Calabash cucumber stalks	Lau sag	*Lagenaria vulgaris*	2.5	90.8
Calabash cucumber	Lau	*Lagenaria vulgaris*	0.9	96.2
Plantain (green)	Kanch kola	*Musa paradisiaca*	1.4	82.8
Water cress	Halim	*Nasturtium officinale*	2.8	85.5
Potato	Alu	*Solanum tuberosum*	1.5	74.5
Jute leaves	Pat sag	*Corchorus olitorius*	2.5	72.8
Drumstick	Saijna danta	*Moringa oleifera*	6.8	75.0

RESULTS AND DISCUSSION

Table 1 presents the samples analysed, their local and botanical names, and their protein percentages. The average results of amino acid composition of 3 samples of each vegetables obtained from different markets are represented in Table 2.

It can be seen from Table 2 that leucines, phenylalaline, valine, r-amino butyric acid, tyrosine, *a*-alanine, threonine, glutamic acid, serine, aspartic acid, cystine, and proline were found to be present in almost all the samples. Excepting methionine, histidine, and arginine, all the other essential amino acids were found in all the samples of vegetables analysed. From the standpoint of essential amino acids, tomato, onion-stalks, onion, drumstick, spinach, cabbage, and neem tender stand first, and then come carrot, beet root, cauliflower, peas, pumpkin, amaranth, cucumber, and potato.

As regards nutritional value, all the samples analysed stand well but amongst them spinach, tomato (green or ripe), onion, drumstick, cabbage, and neem tender should be preferred first.

TABLE 2

Amino acid composition of some common Indian vegetables

Amino acids	Spinach	Carrot	Beet root	Tomato (green)	Tomato (ripe)	Cauliflower	Cabbage	Peas	Pumpkin	Pumpkin stalks	Neem tender	Onion stalks	Onion	Ridge gourd	Radish	Radish stalks	Sweet potato	Amaranth tender	Calabash cucumber stalks	Calabash cucumber	Plantain (green)	Water cress	Potato	Jute leaves	Drumstick
	(Results are expressed in milligrams per gram)																								
Leucines	2.8	4.4	2.7	1.9	0.3	2.6	1.0	5.9	0.9	3.7	3.8	1.3	0.1	0.6	1.3	2.1	1.9	3.5	2.3	0.8	1.2	3.0	2.0	1.4	8.0
Phenylalanine	0.8	0.3	0.3	0.6	0.2	0.6	0.4	3.2	0.3	1.4	2.2	0.07	—	—	—	—	—	0.5	1.0	0.9	—	1.0	0.6	2.3	4.2
Valine	1.1	0.2	1.3	0.4	0.1	1.9	0.5	0.8	0.4	1.7	1.6	1.2	0.02	0.3	0.2	0.8	0.5	1.6	1.0	0.3	1.04	1.2	0.8	2.3	4.0
r-amino butyric Acid	+	+	+	—	+	+	+	+	+	+	+	+	+	—	—	—	+	+	+	+	+	+	—	+	+
Tyrosine	1.0	0.2	—	0.3	0.1	0.5	0.1	1.1	0.2	0.8	1.6	0.2	0.2	0.3	—	0.5	0.1	0.3	0.4	0.4	0.1	0.6	0.3	1.4	2.5
Alanine	1.1	0.2	2.2	1.4	0.2	2.8	0.6	1.9	0.8	0.5	1.7	1.2	1.1	0.3	1.3	0.7	1.06	4.0	1.1	0.5	2.1	1.0	0.7	0.6	3.7
Threonine	1.2	0.3	0.3	0.6	0.3	1.0	0.6	1.8	0.3	1.3	2.3	0.2	0.1	0.3	0.4	0.9	0.4	0.5	0.8	0.2	0.3	1.5	0.7	2.7	4.0
Glutamic Acid	2.9	1.7	2.6	3.5	2.3	2.7	3.8	4.6	1.8	0.8	4.4	0.3	0.9	0.8	1.7	1.6	2.2	4.6	1.7	1.3	2.4	2.7	1.5	1.0	6.2
Serine	—	0.3	0.2	0.6	—	0.1	0.6	0.09	0.4	0.9	1.8	1.3	0.1	0.4	0.4	0.9	0.3	0.6	0.9	0.6	0.2	0.6	0.6	0.5	—
Aspartic Acid	4.2	0.7	1.6	1.7	1.0	2.9	2.8	6.2	2.5	1.4	5.6	0.3	1.3	0.9	0.7	2.1	2.9	3.6	2.9	1.9	1.5	4.0	2.8	2.0	8.2
Cystine	1.9	0.4	0.6	1.2	0.3	2.8	1.2	2.4	0.8	2.9	3.2	0.4	0.4	0.6	1.5	1.5	1.1	2.8	1.7	0.6	0.2	1.0	1.4	3.6	5.1
Cysteine	—	—	—	0.02	—	—	—	—	—	0.7	0.3	0.02	0.1	—	—	—	—	—	0.4	0.3	0.1	—	0.1	—	0.1
Lysine	2.0	0.5	1.2	0.9	0.2	2.1	1.4	4.5	0.6	2.3	3.8	0.3	—	—	—	—	—	0.6	5.2	—	—	1.5	0.7	3.2	1.3
Cystic Acid	—	—	—	+	—	—	—	+	+	+	+	—	+	—	—	—	—	+	+	+	—	—	+	—	+
Glycine	—	—	—	0.6	—	2.4	—	2.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Arginine	—	—	—	—	0.2	—	—	—	—	—	—	—	0.4	0.3	—	0.5	1.1	—	—	0.4	0.3	—	—	—	2.5
Histidine	0.9	—	—	0.2	0.4	—	0.3	—	—	—	0.4	—	—	—	0.9	—	—	—	—	—	—	—	—	—	1.3
Metheonine sulphoxide	—	—	0.05	0.04	—	0.2	—	0.2	0.1	—	0.3	0.04	0.05	—	—	—	—	0.2	0.3	—	0.03	0.1	0.2	0.3	1.2
Proline	0.3	0.1	0.3	0.2	—	0.3	0.2	0.4	0.3	0.4	0.4	0.2	—	—	—	0.3	—	0.6	0.3	0.3	—	0.4	0.5	0.6	3.6
Hydroxy proline	—	—	+	—	—	+	+	+	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ornithine	—	—	—	—	+	+	—	—	—	+	—	—	—	—	+	—	—	+	—	—	—	—	+	+	—
Histamine	—	—	—	—	—	+	+	+	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Unknown	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ denotes the presence of amino acid; — denotes the absence of amino acid.

SUMMARY

Twenty varieties of common Indian vegetables have been analysed for their amino acid contents with the help of paper chromatographic technique. Leucines, phenylalanine, valine, r-amino butyric acid, serine, aspartic acid, cystine, and proline were found to be present in almost all the samples analysed. Tomato, spinach, drumstick, cabbage, neem tender, carrot, beet, cauliflower, peas, pumpkin, amaranth, cucumber, and potato are good sources for essential amino acids. From a nutritional standpoint spinach, tomato, onion, drumstick, cabbage, and neem tender are good sources for both essential and nonessential amino acids both in number and quality.

Acknowledgment

The authors are indebted to Dr. S. C. Roy and Prof. B. C. Guha of the Department of Applied Chemistry, University College of Science and Technology, Calcutta, for their kind suggestions during the course of this investigation.

LITERATURE CITED

1. Bagchi, S. P., Ganguli, N. C., and Roy, S. C. Amino acid composition of some Indian pulses: Quantitative determination by paper chromatography. *Ann. Biochem. & Exptl. Med.*, **15**, 149 (1955).
2. Block, R. J., Durrum, E. L., and Zweig, G. *Paper Chromatography and Paper Electrophoresis.* 1955, Academic Press, New York.
3. Block, R. J., and Weiss, K. W. Studies on bovine whey proteins. IV. The amino acid analysis of crystalline β-Lactoglobulin and α-Lactalbumin by quantitative paper chromatography. *Arch. Biochem. & Biophys.*, **55**, 315 (1955).
4. Block, R. J., and Bolling, D. *The amino acid composition of proteins and foods.* 1951, 2nd ed., C. C. Thomas, Springfield, Ill.
5. Block, R. J., LeStrange, R., and Zweig, G. *Paper Chromatography, a Laboratory Manual.* 1952, Academic Press, New York.
6. Giri, K. V., Radhakrishnan, A. N., and Baidynathan, G. S. Some factors influencing the quantitative determination of amino acids separated by circular paper chromatography. *Anal. Chem.*, **24**, 1677 (1952).
7. Holmes, P. The amino acid composition of certain seed proteins. *Australian J. Exptl. Biol. Med.*, **31**, 595 (1953).

SPECIES OF *CLOSTRIDIUM* ASSOCIATED WITH ZAPATERA SPOILAGE OF OLIVES[a]

TOSHIO KAWATOMARI[b] AND REESE H. VAUGHN
Department of Food Technology, University of California, Davis, California

(Manuscript received March 2, 1956)

"Zapatera," a malodorous spoilage of Spanish green olives apparently first described by Cruess *(3)* in 1924, occurs in all types of brined olives. The off-odor at first is described as "cheesy" or "sagey" but as spoilage progresses it develops into an unmistakable foul, fecal stench.

Under the conditions that prevail in California, "zapatera" spoilage, unlike the butyric acid fermentation already described by Gililland and Vaughn *(6)*, occurs when the desirable lactic acid fermentation is permitted to stop before the pH of the brine has decreased below pH 4.5. At the onset of the spoilage the pH of the affected brine increases while the titratable acidity decreases. Continuous loss in acidity is observed as the spoilage progresses (Ball, *1;* Cruess, *4;* Vaughn, *19;* and Vaughn, Douglas, and Gililland, *20)*.

The cause of "zapatera" is obscure. Smyth *(11)* concluded that it was due "to one or more of a group of spore-forming, proteolytic, facultative rods normally present in the soils of Andalusia." Because the differentiation between true "zapatera" spoilage and butyric acid fermentation is confused in the olive industry, the tendency is to designate all malodorous fermentations as "zapatera." Gililland and Vaughn *(6)* implicated the *Clostridium butyricum* group as the cause of the butyric acid fermentation of olives. The rancid butter odor produced from sugars by these bacteria during the primary stage of fermentation is not true "zapatera" spoilage because the latter results from decomposition of the organic acids at a time when little or no sugar is present. Soriano *(12)* and Soriano and Soriano *(13)* have made the claim that spoilage is caused by the sulfate-reducing bacterium *Desulfovibrio desulfuricans*. However, hydrogen sulfide odor is not pronounced in the case of "zapatera," and so it is doubtful that the sulfate-reducing bacteria are the cause of the spoilage.

Because of the previous inability to isolate bacteria capable of causing "zapatera" it was decided to study the acidic constituents of the brines of fermented olives. Delmouzos, Stadtman, and Vaughn *(5)* found that normal fermented brines contained acetic, lactic, and some succinic acid; whereas, spoiled brines also contained formic, propionic, butyric, valeric, caproic, and caprylic acids. This information suggested the association of

---

[a] The senior author was the recipient of the Samuel Cate Prescott Award of the Northeast Section IFT in June, 1955, for his essay "Spore-forming Obligate Anaerobic Bacteria Associated with 'Zapatera' a Malodorous Fermentation of Olives" which described the preliminary research done on the problem while an undergraduate student. The present paper was presented in part at the Fifteenth Annual Meeting of IFT, Columbus, Ohio, June, 1955.

[b] Present address: Dept. of Bacteriology, 406th Med. Gen. Lab., APO 500, San Francisco, California.

species of the genus *Clostridium* with the malodorous spoilage, especially since previous investigations *(2, 17)* had demonstrated lactate utilization to be accentuated by the presence of acetate. On the basis of these observations an enrichment medium containing acetic and lactic acids was developed and used for isolation and purification of species of *Clostridium* from "zapatera" brines. This study is concerned with the taxonomy of the anaerobic clostridia isolated and with factors which associate them with "zapatera" spoilage.

EXPERIMENTAL METHODS

Source of cultures. Isolation of anaerobic, spore-forming bacteria were made from 54 different samples of brined olives including California green olives (Spanish type), Sicilian type California green olives, and brined olives in storage for California ripe olives, as well as *bona fide* Spanish green olives and brined Greek olives.[c] The majority of the imported olive samples were obtained through the courtesy of the California State department of Health, Bureau of Food and Drug Inspections. A few imported Spanish green olive samples and the rest of the California samples (Spanish-type, Sicilian-type, and storage olives) were obtained directly from the California industry by the authors alone or in cooperation with representatives of the California Olive Advisory Board.

The most productive isolation and purification medium (several were investigated) had the following composition:

Sodium acetate	5 g.
Calcium lactate[d]	5 g.
Proteose peptone, Difco	5 g.
Bacto-tryptone	5 g.
Soluble starch	1 g.
Sodium thioglycollate (Mercaptoacetic acid salt)	0.5 g.
Agar for semi-solid medium	5 g.
or	
Agar for solid medium	20 g.
Distilled water to make 1 liter	

The reaction of the medium was adjusted to pH 6.5 before sterilization for 15 minutes at 121° C. (250° F.). The pH of the medium after sterilization varied between 5.7 and 6. The suitability of this medium for enrichment and purification of the clostridia was shown by the rapid, vigorous growth in the original enrichment which was sustained on subsequent transfer and storage.

Enrichment cultures always were made in duplicate. First, in order to neutralize the acidity of the suspected brines, screw-capped 25 × 200 mm. tubes were prepared to contain 0.5 g. of calcium carbonate and sterilized. Then 10-ml. portions of the spoiled brines were added aseptically to these tubes. The brines thus neutralized then were heated to 80° C. (176° F.) and held for 5 minutes. After the brines were cooled to room temperature 1-ml. aliquots were transferred to sterile 15 × 200 mm. Veillon tubes which contained 0.5 ml. of sterile 5% sodium bicarbonate solution to foster rapid spore germination. Then freshly prepared sterile semi-solid acetate-lactate medium was poured aseptically into each inoculated Veillon tube until it was three-fourths full. The tube finally was sealed with melted "vaspar." Each large tube containing the remainder of the heated sample (9 ml.) was filled with medium in the same manner but not sealed. The inoculated tubes then were incubated at 30° C. (86° F.).

---

[c] For an understanding of the various types of olives and their processing consult Vaughn *(19)*.

[d] Calcium lactate is more convenient but lactic acid neutralized with NaOH gives a clearer medium which is desirable for purification of cultures.

Spores of acetate-lactate fermenting species soon germinated and, after formation of many vegetative cells, caused copious gas production in the enrichment medium; some within 9 hours after inoculation. Positive enrichment cultures, whether from the 1-ml. or the 9-ml. original sample, were given four successive enrichments by transfer of 0.5 to 1 ml. of each positive culture by Pasteur pipette into the acetate-lactate medium in Veillon tubes in order to minimize aerobic bacteria.

Isolation and purification of cultures were started from the fourth positive enrichment culture. A drop or two of inoculum from the depths of the culture was placed on the surface of the solid acetate-lactate medium contained in porous-top plates and streaked with a loop. The streaked plates were incubated in McIntosh and Fildes anaerobic jars in an atmosphere of 95% N_2 and 5% CO_2 for 4 or 5 days at room temperature with the usual precautions to maintain anaerobiosis.

Two to three different morphological colony types frequently were found on the same plate. Each type was picked separately and inoculated into the acetate-lactate medium contained in a Veillon tube which was incubated at 30° C. (86° F.). Each culture that grew then was restreaked until it was considered to be pure.

The chief difficulty encountered in the isolation and purification of the 270 cultures by the procedure described was the detection and separation of slow-growing cultures that had been overgrown by more vigorous forms. In some cases prolonged incubation (up to 2 weeks) was necessary to be sure to detect mixtures of the two types.

CHARACTERISTICS OF THE ISOLATES

The criteria used for identification of the cultures followed the recommendations of Spray *(15)*, Reed and Orr *(10)*, and McClung *(7)*. Special emphasis was placed on: form and position of the spores; decomposition of coagulated egg albumin; liquefaction of gelatin; production of indol; fermentation of carbohydrates including glucose, lactose, sucrose, salicin, glycerol, mannitol, and starch; pigment production in corn meal mash medium; nitrate reduction; production of hydrogen sulfide; and coagulation and digestion of iron-milk medium. Supplementary observations concerning morphological differences and ability to liquefy polypectate medium (Nortje and Vaughn, *8)* also were used.

An attempt was made to allocate the 270 obligate anaerobes to species already recognized by Spray in *Bergey's Manual of Determinative Bacteriology (16)*. The characteristics of 231 isolates permitted ready identification as shown in Table 1. The remaining 39 cultures differed in a few characteristics (one to four) from recognized species as shown in Table 2.

C. sporogenes (138 cultures) was the most abundant species isolated and identified. The second most abundant type, *C. bifermentans,* was represented by 77 isolates. The other identifiable species *C. butyricum, C. multifermentans, C. beijerinckii, C. pasteurianum,* and *C. sphenoides,* represented by only 19 cultures inclusively, were in the minority. With but 1 exception *(C. cochlearium* ?), the remaining unidentified cultures (Table 2) were saccharolytic types, probably closely related to the large "butyricum" group of anaerobic sporeforming bacteria. These observations indicate that the more abundant proteolytic-saccharolytic species *C. sporogenes* and *C. bifermentans* may be the most important ones associated with "zapatera" spoilage of olives. However, as shown in Table 3, *C. bifermentans* was recovered from 38 different samples whereas *C. sporogenes* was isolated from only 22 samples. Therefore, it may be that *C. bifermentans* is the predominant species associated with "zapatera" spoilage even though more cultures of *C. sporogenes* were isolated.

TABLE 1

Characteristics of the readily identified species of Clostridium isolated from "zapatera" olive brines

Number of cultures	Spore morphology			Proteolysis			Carbohydrate fermentation							Other reactions				Iron-milk medium	Species allocation
	Shape	Position	Sporangium swollen	Liquefaction of coagulated egg albumin	Gelatin liquefaction	Indol production	Glucose	Lactose	Sucrose	Salicin	Glycerol	Starch	Mannitol	Pigment production	Polypectate liquefaction	NO_3 reduced to NO_2	H_2S (Pb Ac, Spray, 15)		
	e	f	g	Number of cultures showing positive reactions at 35° C. (95° F.)														h	
4	Ov	St	+	0	0	0	4	4	4	4	0	4	4	0	0	0	4	A	*C. butyricum*
5	Ov	St	+	0	0	0	5	5	5	5	5	5	0	0	2	0	5	A	*C. multifermentans*
3	Ov	St	+	0	0	0	3	3	3	3	0	0	3	0	0	0	3	A	*C. beijerinckii*
1	Ov	St	+	0	0	0	1	0	1	1	0	0	1	0	0	0	1	A	*C. pasteurianum*
3	S	St	+	3	0	0	3	0	3	3	0	0	3	0	0	0	3	I	*C. sphenoides*
138	Ov	St	+	138	138	0	138	3	0	0	68	0	0	0	0	0	138	D	*C. sporogenes*
77	Ov	St	−	77	77	77	77	0	0	23	77	0	0	0	0	0	77	D	*C. bifermentans*

[e] Ov = oval, S = spherical.
[f] St = subterminal.
[g] + = swollen, − = not swollen.
[h] A = Active gaseous fermentation.
I = Inactive gaseous fermentation.
D = Digestion.

TABLE 2

Characteristics of the unidentified clostridia isolated from "zapatera" olive brines

Number of cultures	Spore morphology			Proteolysis			Carbohydrate fermentation							Other reactions					Possible species allocation	Exceptions which prevent ready speciation
	Shape	Position	Sporangium swollen	Liquefaction of coagulated egg albumin	Gelatin liquefaction	Indol production	Glucose	Lactose	Sucrose	Salicin	Glycerol	Starch	Mannitol	Pigment production	Polypectate liquefaction	NO_3 reduced to NO_2	H_2S (PbAc, Spray)	Iron-milk medium		
	e	f	g	Number of cultures showing positive reactions at 35° C. (95° F.)														h		
17	Ov	St	+	0	0	0	17	0	0	0	17	17	0	0	0	0	17	I	*C. novyii*, type 1 †	gelatin not liquefied
5	Ov	St	+	0	0	0	5	0	0	0	5	0	0	0	0	0	5	I	*C. novyii*, type 2 †	gelatin not liquefied, starch not attacked
1	Ov	St	−	0	0	1	1	0	0	0	1	0	0	0	0	0	1	I	*C. novyii*, type 3 †	Sporangium not swollen; gelatin not liquefied; starch not attacked; indol produced
2	S	St	+	0	0	0	2	2	2	2	2	2	2	0	0	0	0	A	*C. butyricum* †	Spherical spores; glycerol fermented; NO_3 not reduced to NO_2
8	Ov	St	+	0	0	0	8	8	8	8	0	8	0	0	0	0	0	I	*C. paraputrificum* †	Subterminal spores
2	Ov	T	+	0	0	0	2	2	2	2	0	2	2	0	0	0	2	I	*C. tetrium* †	NO_3 not reduced to NO_2; not microaerophilie
1	S	T	+	0	0	1	1	0	0	0	0	0	0	0	0	0	1	I	*C. tetanomorphum* †	H_2S produced
3	Ov	St	−	0	0	0	0	0	0	0	0	0	0	0	0	0	3	I	*C. cochlearium* †	Coagulated egg albumin softened in 30 days. H_2S produced in 10 days

[e] See footnote Table 1.
[f] See footnote Table 1.
[g] See footnote Table 1.
[h] See footnote Table 1.

TABLE 3

Frequency of isolation of C. bifermentans and other species from 54 samples of "zapatera" olive brines

Species isolated	Number of samples
C. bifermentans alone	8
C. sporogenes alone	0
C. bifermentans and *C. sporogenes*	20
C. bifermentans, *C. sporogenes*, and other species	2
C. bifermentans and species other than *C. sporogenes*	8
C. multifermentans alone	1
Unidentified cultures	8
No isolates	7
Total	54

MAXIMUM SALT TOLERANCE

Preservation of olives by fermentation is dependent upon the concentration of salt as well as the acidity of the brines. Most olives are fermented in brines containing about 5 to 8% salt and, after fermentation, may be stored in brines with as much as 15% sodium chloride (Cruess, *4;* Vaughn, *19;* and Vaughn et al., *20).*

To test the resistance of the "zapatera" clostridia to salt the cultures were grown in the semi-solid acetate-lactate medium at 35° C. (95° F.). After the cultures grew vigorously they were first transferred into the organic acid medium containing 1.0% NaCl. When growth was observed in the test medium, a portion (0.1 to 0.2 ml.) of the culture was transferred to the medium with 2.0% salt. This adaptation technique was continued with increasing concentrations of salt in 1.0% increments until the majority of cultures failed to grow. Results of these tests are shown in Table 4.

All of the cultures grew in the presence of 1.0% salt and most of them tolerated 2 and 3% salt. Twenty-three of the 39 cultures grew in the presence of 4.0% salt; 18 resisted 5% salt; 13 tolerated 6% salt, and 7 tolerated 7% salt. Only 4 isolates of *C. sporogenes* grew in the medium with 8% salt. These same 4 cultures failed to develop in the presence of 9% salt. The resistance of these test cultures, particularly those belonging to the "butyricum" complex, corresponds with observations previously recorded by Gililland and Vaughn *(6).* However, it is interesting to note that the cultures of *C. sporogenes* and *C. bifermentans* showed greater resistance to salt than the majority of the other types tested.

MINIMUM pH TOLERANCE

It has been known for some time that a few of the butyric acid anaerobes can grow in rather acid surroundings (Townsend, *18;* Spiegelberg, *14;* and Gililland and Vaughn, *6).* It is common practice in the olive industry (in California at least) to attempt to control the fermentation until the pH of the brine has decreased to about 3.8 in order to avoid "zapatera" spoilage. To test this practical observation the "zapatera" cultures were tested for minimum pH tolerance. Liver infusion broth was used as the test medium because it had been determined that the acetate-lactate medium,

TABLE 4

Sodium chloride resistance of some of the clostridia isolated from "zapatera" olive brines

Bacteria	Number tested	NaCl, grams per 100 ml.[1]							
		1	2	3	4	5	6	7	8[2]
Identified species of *Clostridium*									
C. butyricum	1	1	1	1	1	1	0	0	0
C. multifermentans	3	3	3	3	3	2	0	0	0
C. beijerinckii	2	2	2	2	2	0	0	0	0
C. pasteurianum	1	1	1	1	1	0	0	0	0
C. sphenoides	1	1	1	0	0	0	0	0	0
C. sporogenes	6	6	6	6	6	6	6	5	4
C. bifermentans	7	7	7	7	7	7	6	1	0
Unidentified types of *Clostridium*									
C. novyii, type 1 ?	7	7	7	6	0	0	0	0	0
C. novyii, type 2 ?	4	4	4	3	1	0	0	0	0
C. novyii, type 3 ?	1	1	1	1	1	1	0	0	0
C. paraputrificum ?	3	3	2	0	0	0	0	0	0
C. tertium ?	1	1	1	1	1	1	1	1	0
C. cochlearium ?	2	2	2	2	0	0	0	0	0
Total	39	39	38	33	23	18	13	7	4

[1] Contained in the semi-solid acetate-lactate medium.
[2] None of the cultures tolerated 9.0% salt.

even when supplemented with 0.1% glucose and 0.5% yeast extract, did not favor the growth of the bacteria at a pH below 5.5. The test media were prepared by adding appropriate amounts of McIlvaines' buffer solutions (0.2 *M*. Na_2HPO_4 and 0.1 *M*. citric acid) to portions of double-strength liver infusion with 1% Bacto-tryptone. This addition diluted the test medium to single strength at the desired pH level. The media then were tubed with dried liver particles and autoclaved for 20 minutes at 121° C. (250° F.). The pH values of the sterilized media were as follows: 6.0, 5.5, 5.0, 4.8, 4.7, 4.6, and 4.5, + or –0.05.

To ascertain the minimum pH tolerance the selected test cultures were grown in the liver infusion medium adjusted to pH 6.0. When the cultures grew vigorously they were transferred (0.1 to 0.2 ml. portions) in duplicate to the medium adjusted to pH 5.5. In this manner the cultures were subjected to ever decreasing pH values until there was no visible sign of growth. Results are shown in Table 5.

All of the cultures grew well in the medium at pH 6.0. Most of them (31) grew in the medium adjusted to pH 5.5. At the lower values the effect of pH became more apparent. Only 23 cultures grew at pH 5.0 and 18 at pH 4.8. When the pH was reduced to 4.7, only 9 cultures grew. At pH 4.6 the number was decreased to 4 and at pH 4.5, the lowest value tolerated by any of the isolates tested, only 1 culture of *C. pasteurianum* could grow.

REPRODUCTION OF "ZAPATERA" SPOILAGE

Satisfactory reproduction of the malodorus emanations of "zapatera" spoilage was not easy. Preliminary experiments with sterile laboratory

TABLE 5

pH tolerance of some of the clostridia isolated from "zapatera" olive brines

Bacteria	Number tested	pH of liver infusion medium						
		6.0	5.5	5.0	4.8	4.7	4.6	4.5
		Number producing gas						
Identified species								
C. butyricum	2	2	2	2	2	2	1	0
C. multifermentans	2	2	2	2	2	1	1	0
C. beijerinckii	2	2	2	2	2	2	0	0
C. pasteurianum	1	1	1	1	1	1	1	1
C. sphenoides	1	1	1	1	1	0	0	0
C. sporogenes	6	6	6	6	6	2	1	0
C. bifermentans	6	6	6	6	2	1	0	0
Unidentified types								
C. novyii, type 1 ?	5	5	3	0	0	0	0	0
C. novyii, type 2 ?	4	4	3	0	0	0	0	0
C. novyii, type 3 ?	1	1	1	1	1	0	0	0
C. paraputrificum ?	3	3	3	2	1	0	0	0
C. tertium ?	1	1	1	0	0	0	0	0
C. cochlearium ?	2	2	0	0	0	0	0	0
Total	36	36	31	23	18	9	4	1

fermented olive brines neutralized to pH 5.5 or above were unsuccessful although a series of trials were made with a variety of cultures.

The characteristic odors were reproduced only when the neutralized brines were supplemented with 0.1% glucose, 0.5 Bacto-peptone, and 0.5% Bacto-yeast extract. However, even with this supplementation, spoilage was consistent only when young, actively growing cultures were used as the inoculum.

The need for this supplementation is not surprising in view of the complex general nutritional and spore germination requirements of members of the genus *Clostridium*. The inactivity of the anaerobes in the sterile, readjusted, laboratory fermented brines suggests that unknown, nutritive, accessory compounds are not readily available or are lacking or that inhibitory substances which hinder the germination of the spores are formed by neutralization or sterilization. In commercial fermentations, considerable yeast film may develop on the olive brines. Autolysis of these yeasts would provide at least a part of the preformed nutrients supposedly not available in the carefully controlled laboratory fermented olives used in this study. Too, a succession of groups of bacteria might produce the preformed accessory nutritive compounds required by the clostridia. Whatever the cause, additional investigations are required to explain the discrepancies. These studies are under way.

DISCUSSION

The association of species of *Clostridium* with "zapatera" spoilage of olives does not give a complete explanation as to the etiology of the spoilage. Propionic acid is one of the abnormal volatile acids found in the spoiled brines. It is obvious, as already suggested by Delmouzos, Stadtman, and Vaughn *(5)* that this acid might arise either through the activity of *C.*

propionicum or the true propionic acid bacteria. *C. propionicum* was not identified in this study. Therefore, an additional investigation was initiated to determine whether species of *Propionibacterium* also might be associated with the spoilage. Plastourgos and Vaughn *(9)* have found propionic acid bacteria to be abundant in "zapatera" brines. This observation suggests that "zapatera" is not caused by a specific agent but is a manifestation of nature's attempt to cause mineralization of the olives. If this assumption is true, further study may associate other microorganisms with the spoilage.

SUMMARY

The characteristics of 270 cultures of *Clostridium* isolated from "zapatera" olive brines are given. Two saccharolytic-proteolytic species, *C. bifermentans* and *C. sporogenes*, predominated among the cultures isolated. Representative isolates caused malodorous fermentation of olive brines when suitable conditions for growth were maintained.

The differential characters for the identified species (231 isolates comprising 7 species) were in good agreement with previous descriptions. However, 39 cultures were not identified because their characteristics did not agree with those of accepted species.

It is suggested that the species of *Clostridium* studied are but one of a number of groups of microorganisms involved in the malodorous "zapatera" spoilage of olives.

Acknowledgments

The authors are indebted to Dr. L. S. McClung for the gift of cultures of known species of *Clostridium* and to Mr. George K. York II for technical assistance. Support of this work with funds from the State of California Olive Advisory Board also is gratefully acknowledged.

LITERATURE CITED

1. Ball, R. N. Seventeenth Annual Technical Conference, *California Olive Assoc. Proc.*, (1938) mimeograph, p. 30.
2. Bhat, J. V., and Barker, H. A. *Clostridium lacto-acetophilum* nov. spec; and the role of acetic acid in the butyric fermentation of lactate. *J. Bact.*, **54**, 381 (1947).
3. Cruess, W. V. Olive pickling in Mediterranean countries. Calif. Agr. Exp. Sta. *Circ. 278* (1924).
4. Cruess, W. V. Olive products. *Ind. Eng. Chem.*, **33**, 300 (1941).
5. Delmouzos, J. G., Stadtman, F. H., and Vaughn, R. H. Malodorous fermentation, acidic constituents of zapatera olives. *J. Agr. Food Chem.*, **1**, 333 (1953).
6. Gililland, J. R., and Vaughn, R. H. Characteristics of butyric acid bacteria from olives. *J. Bact.*, **46**, 315 (1943).
7. McClung, L. S. On the enrichment and purification of chromogenic spore-forming anaerobic bacteria. *J. Bact.*, **46**, 507 (1943).
8. Nortje, B. K., and Vaughn, R. H. The pectolytic activity of species of the genus *Bacillus:* Qualitative studies with *Bacillus subtilis* and *Bacillus pumilus* in relation to the softening of olives and pickles. *Food Research*, **18**, 57 (1953).
9. Plastourgos, S., and Vaughn, R. H. (Unpublished data, 1956.)
10. Reed, G. B., and Orr, J. H. Rapid identification of gas gangrene anaerobes. *War Med.*, **1**, 493 (1941).
11. Smyth, H. F. A bacteriologic study of the Spanish green olives. *J. Bact.*, **13**, 56 (1927).

12. SORIANO, S. La bacteria causante de la aceituna "zapatera" y su control. Primera Conferencia Nacional de Olivcultura. Corporacion Nacional de Olivicultura. Commission Ejecutive. Buenos Aires. 397 (1955).
13. SORIANO, S., AND SORIANO, A. M. DE. Estudio microbiologico de una alteracion de aceitunas conservadaes en salmuera. *Rev. asoc. Argentina dietol.*, **4**, (13), 132 (1946).
14. SPIEGELBERG, C. H. *Clostridium pasteurianum* associated with spoilage of an acid-canned fruit. *Food Research*, **5**, 115 (1940).
15. SPRAY, R. S. Semisolid media for cultivation and identification of the sporulating anaerobes. *J. Bact.*, **32**, 135 (1936).
16. SPRAY, R. S. In Bergey's Manual of Determinative Bacteriology, 1948, 6th ed., The Williams and Wilkins Co., Baltimore, p. 763.
17. TABACHNICK, J., AND VAUGHN, R. H. Characteristics of tartrate-fermenting species of *Clostridium*. *J. Bact.*, **56**, 435 (1948).
18. TOWNSEND, C. T. Spore-forming anaerobes causing spoilage in acid-canned foods. *Food Research*, **4**, 231 (1939).
19. VAUGHN, REESE H. "Olives and Pickles," Vol. 4, Part II, 1st ed., Quartermaster Food and Container Institute for the Armed Forces (1946).
20. VAUGHN, R. H., DOUGLAS, H. C., AND GILILLAND, J. R. Production of Spanish-type green olives. Calif. Exp. Sta. *Bull. 678* (1943).

STRAIN RETARDATION IN STARCH JELLY CANDY

CLARENCE STERLING
Department of Food Technology,
University of California, Davis, California

(Manuscript received March 13, 1956)

The formation of a starch gel is believed generally to involve the parallel association of linear parts of starch molecules at "junction points" or micelles. Extensions of the molecules from micelle to micelle form the connecting links of an irregular network (*14*). Because of this reticulate structure, the starch gel presumably can show elastic behavior when it is stressed.

However, in most polymer gels, the elastic properties are not perfect. During a period of stress, only a small portion of the strain is reached instantaneously (*i.e.*, with sonic speed). Further strain is attained gradually, over a sensible period of time. Similarly, when the stress is released, the recovery takes place within a noticeable time interval. These phenomena constitute "strain retardation" (*16*). Often the recovery is incomplete; some permanent deformation may remain. Mechanical models have been of considerable help in analyzing the course of the strain-time relationship, and many workers have relied on these mechanical analogues in deriving appropriate equations for the behavior of their materials (*1, 13, 15*).

Much of this prior work has been performed on industrial materials (*e.g.*, rubber, modified cellulose fibers, synthetic fibers, etc.). Pioneering studies on rheological properties of dough have been made by Scott Blair and his associates (*8, 9, 18, 19, 20*) and Hlynka *et al.* (*3, 5*). In general, however, food materials have not been investigated as intensively as the industrial commodities. The present study proposes a detailed consideration of the rheological behavior of starch jelly candy from the standpoint of strain retardation.

Materials and Methods

The starch jelly was prepared as follows: 25 g. of sucrose, 25 g. of dextrose, and 50 g. of 42 D. E. corn syrup were added to 55 ml. of distilled water, and the mixture was brought to a boil, during which process the ingredients dissolved in the water. The solution was stirred continuously while a freshly agitated slurry of 12 g. of acid-modified corn starch (60 fluidity) suspended in 55 ml. of water was added. Stirring continued until the starch was gelatinized, at which point the force of ebullition agitated the candy mixture.

When the mixture had been boiled down to a weight of 137 g., it was removed from the heat and further mixed with 2 ml. of buffer solution (4 parts 0.9 M citric acid; 1 part 1.0 M Na_2HPO_4) and 0.25 ml. lemon oil. This final mixture was poured into cylindrical starch molds (1.27 cm. diameter x 2.90 cm. length) and the jelly allowed to set at room temperature, shrinking to a length of 2.60 cm. in 24 hours. Since gel-setting occurs within 24 hours, this age of jelly was chosen for the trials of the present study.[a]

[a] In usual commercial practice, setting and evaporation of water from the jelly takes place in a heated room (about 130°F.). However, the formulation used here permitted setting at room temperature, which restricted water loss (and hence shrinkage) of the jelly.

Several series of candies were used for the measurements. Candies were stressed in compression for 20 minutes with 2, 5, 10, 20, 50, and 100 g. weights, respectively. (A fresh sample was used for each determination.) At the end of the 20-minute period, the weight was removed and the candy allowed to recover for 20 minutes. By means of the apparatus described earlier (*2*), the curves of deformation against time (during loading and after the removal of load) were recorded continuously.

Deformation was converted into strain by using the equation (see *21*),

$$\sigma = \ln\left(\frac{l_o}{l}\right), \tag{1}$$

where σ is the strain ($\sigma \times 100$ = percentage strain), and the right-hand term is the natural logarithm of the original length divided by the deformed length, on the basis of $\sigma = \int_{l}^{l_o} \frac{dl}{l} = \ln\left(\frac{l_o}{l}\right)$. If it be assumed that Poisson's ratio for this material is virtually 0.5 (*i.e.*, that there is scarcely any decrease in volume during compression), the minimum stress at any deformation can be calculated from the equation,

$$S = \frac{Wt \times l \times 980}{V}, \tag{2}$$

where S = stress in dynes/cm.^{2}

Wt = weight (in grams) pressing on candy

$$\frac{l}{V} = \frac{\text{length}}{\text{volume}} = \frac{1}{\text{cross-sectional area (in cm.}^2\text{)}}$$

980 = factor to convert grams of force to dynes

or
$$S = Wt \times l \times 300 \tag{3}$$

(since $V = 3.27$ cm.^{3}).

Results and Discussion

Mechanical analogies. The representative data for strain and time at different loads are given in Table 1. (The general form of the curve of strain against time for compression and release is shown for the 50 g. load in Figure 1.) Since the stress decreases as the cross-sectional area increases (with increasing strain), the presumptive actual relationship for strain

TABLE 1

Representative values of strain in compression and recovery under different loads

Time (sec.)	Strain (%) in compression Load (g.) on					
	2	5	10	20	50	100
18	1.55	4.28	7.89	13.37	22.23	32.61
35	1.77	4.52	8.35	13.94	23.21	33.45
60	1.92	4.79	8.61	14.71	23.81	33.92
300	2.22	5.19	9.21	15.65	25.08	35.48
600	2.28	5.35	9.45	15.83	25.43	35.97
1200	2.29	5.51	9.64	16.04	25.83	36.47
	Strain (%) in recovery Load released					
18	1.12	2.04	3.32	5.35	10.12	15.50
35	1.03	1.88	2.99	4.96	9.34	14.67
60	0.95	1.78	2.77	4.56	8.89	14.02
300	0.77	1.49	2.22	3.88	7.51	12.70
600	0.70	1.39	2.04	3.66	7.13	12.15
1200	0.58	1.31	1.90	3.33	6.79	11.76

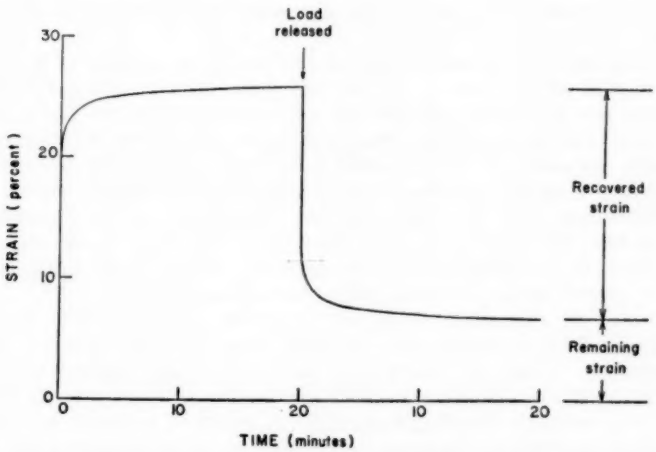


Figure 1. Loading-recovery curve of starch jelly under 50-g. load. Load applied at 0 minute.

against time at a given stress can be approximated by interpolation. When the strain is plotted against the calculated stress for given times, the curves of strain against time may be redrawn for given stresses. The re-plotted values are somewhat higher than the original values of the strain (by a maximum of 2.5% for the 20-minute determination with 100 g.). Because these differences are relatively small, because they are distributed proportionally, because the form of the curves is therefore the same, and because of the uncertainties of graphical interpolation, there does not appear to be

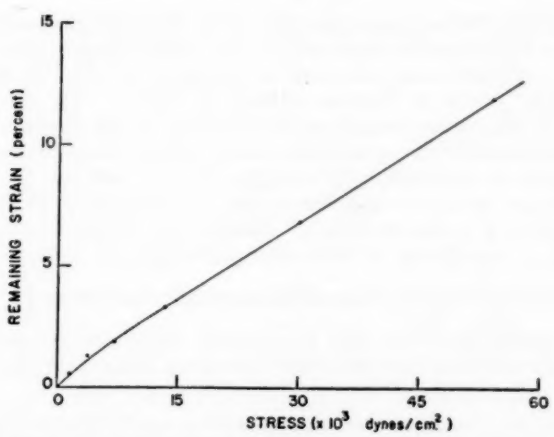


Figure 2. Amount of strain remaining after 20 minutes of recovery. Stress values are those at 20 minutes of loading.

excessive license in the use of the unmanipulated stress-strain determinations.

(Parenthetically, it is necessary to discuss the assumption of a Poisson ratio of 0.5. At the end of the first 24 hours of gel-setting, the candy cylinders are still quite soft and are deformed and crushed under rather small pressures. Even though some moisture is lost during the 24 hours in the mold, the moisture content is still quite high — about 22%. In addition, measurements on the compressed gel indicate little volumetric change from the unstrained state. Finally, it should be pointed out that should the Poisson ratio be significantly lower than 0.5, there would be less change in the values of the stress during compression, which would lead to even smaller corrections in the strain values than indicated above.)

Because the original length is not completely recovered after release of the stress (Table 1), it may be speculated that "flow" has occurred during compression. When the amount of unrecovered deformation is plotted against the stress, a nearly rectilinear relationship is found (Fig. 2). On the basis of a mechanical model, it could be appropriate to depict a "viscous" element (*e.g.*, dashpot) linked in series with the other elements. The value of "η" in poises would be given by the relationship,

$$\text{"}\eta\text{"} = \frac{\mathbf{S}}{3\,\frac{d\sigma}{dt}} \tag{4}$$

where "η" = coefficient of "viscosity" in poises

$\frac{d\sigma}{dt}$ = rate of strain = $\frac{\text{remaining deformation}}{\text{1200 seconds}}$

$\frac{\mathbf{S}}{3}$ = shear stress ($\frac{1}{3}$ compressive stress, where Poisson ratio is 0.5).

On this basis, the average value for "η" is 1.8×10^8 poises. However, a slight concavity toward the stress axis at the smaller stresses (up to 10×10^3 dynes/cm.^{2}) indicates some dilatancy in this region (*17, 21*). In other words, the "viscosity" tends to increase slightly at first, as the rate of shear increases. No simple mechanical model is known for the dilatant feature.

The relationship of the completely elastic portion of the 20-minute strain with the stress is somewhat more complex (Table 1 and Fig. 3). This relationship is not rectilinear and hence does not describe a Hookean body ($\mathbf{S} = \mathbf{E}\sigma$ where $\mathbf{E}$ is the modulus of elasticity, *i.e.*, Young's modulus). The relationship is curvilinear in both semilogarithmic and double-logarithmic graphing. Nevertheless, if the value of the modulus for each stress $\left(\frac{\mathbf{S}}{\text{recovered } \sigma}\right)$ is plotted against the stress (Fig. 4), a nearly rectilinear relation is found.[b] In brief, the modulus increases with increasing stress — or, as the stress

[b] It must be recognized that this calculation lacks theoretical justification: (1) Young's modulus for an elastic material is usually computed on the basis of the total strain; and (2) both total and recoverable strain are conceivably time-dependent. Nevertheless, this relationship is usually assumed in mechanical models. (Also see *4, 8*)

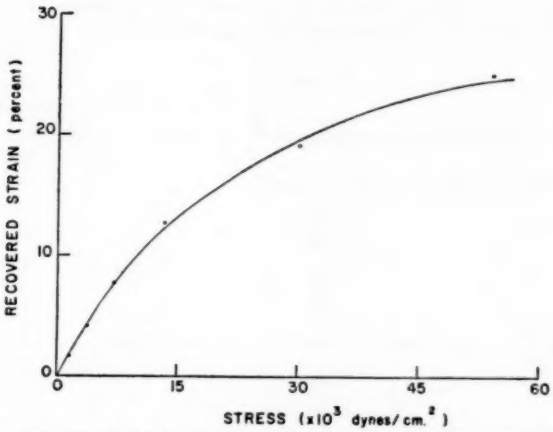


Figure 3. Amount of strain recovered after 20 minutes of recovery. Stress values are those at 20 minutes of loading.

increases, the amount of strain per unit stress decreases. [If the modulus be plotted against the strain, there is a curvilinear relationship (convex to the strain axis) with sharp increases in the modulus at higher strains.] This effect could be represented by a single helical spring of continuously increasing wire diameter. At the intercept on the modulus axis, the Young's modulus, in recovery, of the unstrained candy is 7.8×10^4 dynes/cm.^{2}

Inasmuch as the recovery of strain is temporally retarded, it becomes necessary to couple this variable modulus unit in parallel with a viscous type of element, as in the so-called Kelvin body (*16*) or Voigt model (*1*). The theoretical Kelvin body consists of a spring and a dashpot in parallel. Its viscous element thereby retards the immediate attainment of full strain $\left(\frac{S}{E}\right)$ on

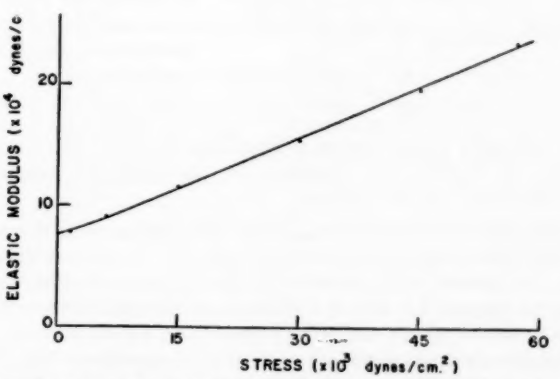


Figure 4. Elastic modulus, based on 20-minute stress divided by recovered strain. Stress values are those at 20 minutes of loading.

compression or of original length on removal of the stress. The equations governing the behavior of this body are as follows:

$$S = n\sigma + \eta \frac{d\sigma}{dt}$$

where n = the shear modulus $\left(\frac{E}{3}\right)$.

If the stress is removed, $S = 0$, and

$$\eta \frac{d\sigma}{dt} = -n\sigma$$

$$\frac{d\sigma}{dt} = -\frac{n}{\eta}\sigma.$$

Letting $\frac{\eta}{n} = \tau_{ret}$ (time of retardation), a constant,

$$\frac{d\sigma}{dt} = -\frac{\sigma}{\tau_{ret}}. \tag{5}$$

If the equation be developed further,

$$\frac{d\sigma}{\sigma} = -\frac{dt}{\tau_{ret}}.$$

$$\text{Integrating, } \ln\sigma - \ln\sigma_o = -\frac{t}{\tau_{ret}}$$

$$\text{or} \qquad \ln\left(\frac{\sigma}{\sigma_o}\right) = -\frac{t}{\tau_{ret}}$$

Thus, in the Kelvin body, time is seen to be a linear function of the logarithm of the strain. However, this type of relationship does not occur in the starch jelly. During the first 60-100 seconds of stressing (and of recovery), the strain-time relationship is of the form,

$$\sigma = a \ln t + K \text{ (fig. 5)},$$

where a = slope of the line.

$$\text{Differentiating, } d\sigma = a\frac{dt}{t}$$

$$\text{or} \qquad \frac{d\sigma}{dt} = \frac{a}{t}. \tag{6}$$

Hence, the early rate of strain is inversely proportional to time, as contrasted with the Kelvin body (equation 5), in which the rate of strain is directly proportional to the strain.

No simple mechanical model can be adapted to describe the relationship of equation 6. Combining the element of variable modulus with a viscosity unit in parallel preserves the form of the Kelvin body relationship. It seems reasonable to suggest the use of a thixotropic system of some type as the simplest component which might be coupled (in parallel) with the elastic unit. A thixotropic unit would show a time dependency like that of the empirical equation 6. However, the thixotropic model would require the use of springs as well as dashpot, since there is an increase in yield value (*17*)

with time (*i.e.*, amount of stress required to initiate flow). Goodeve and Whitfield (7) have suggested the dependence of the apparent viscosity of a thixotropic system on the rate of shear and have indicated the rôle of the yield value in this system. No satisfactory equations are available, from which to construct a thixotropic model.

The type of curve of Figure 5 can be used to estimate the instantaneous

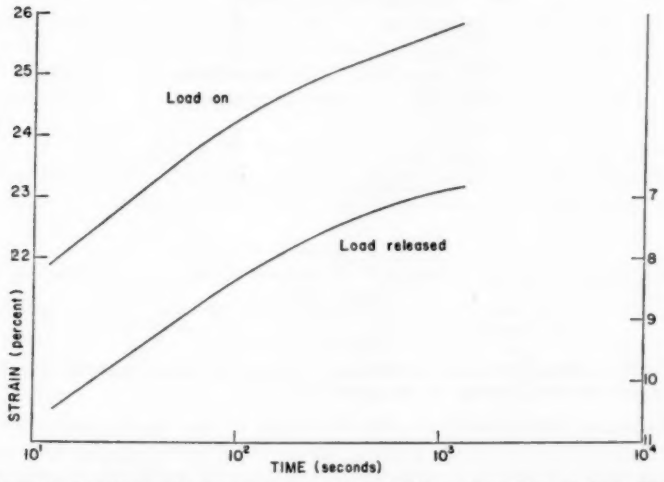


Figure 5. Loading and recovery curves (50-g. load) vs. logarithm of time. Recovery curve values are to be read from right-hand side of graph.

elastic strain (and thereby the static Young's modulus of the material) by projecting the straight line at the beginning of the curve to 10^{-2} second as an approximation (see *15*). When this extrapolation is performed and the modulus computed, the values for most of the curves range closely about an average of 2.9×10^5 dynes/cm.^{2}. This value could account for a part of the early, very rapid deformation and the early, rapid recovery of the candy on stress and release, respectively.

Coupling the instantaneous elastic element and the viscous dashpot in series forms a Maxwell body (*1, 16*), within which may be coupled the postulated anomalous Kelvin body. This form of unit is regarded by Alfrey (*1*) as representing the simplest type of plasto-elastic behavior. Although the above model (Fig. 6) might "explain" the gross behavior of the creep and recovery curves, much of the detail of the curves can not be fitted readily by the simple model. Various workers (*1, 11, 13, 15*) have preferred to regard creep curves as reflecting continuously varying retardation times in their materials. In other words a spectrum of retardation times is considered to exist, based on degrees of kinking and curling and on kinds and extents of bonding between molecules. Models for such distributions of retardation times are necessarily quite complex. Graphical methods of constructing distribution curves of retardation times have been suggested by Alfrey (*1*) and used by Cunningham *et al.* (*3*).

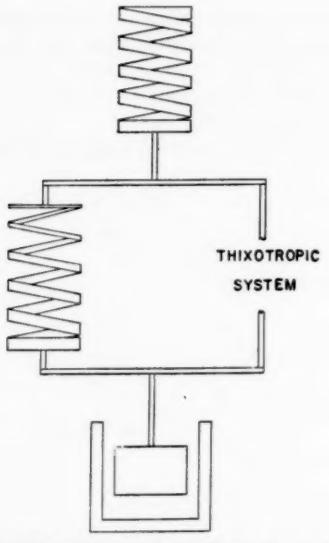


Figure 6. Mechanical model which might duplicate the major features of 20-minute loading and recovery behavior of the starch jelly.

Molecular interpretation. On the basis of the mechanical analogies, some clues are given for interpreting the presumptive molecular behavior of the gel during stress and recovery. As a starting point, the starch gel is considered to be organized in a reticulum, in which the junction points are either polysaccharide micelles or hydrogen bridges (through sugar and water molecules). Unbonded or lightly bonded starch and dextrin molecules are probably present also, scattered in a "brush-heap" arrangement through the reticulum.

The nearly instantaneous large deformation on stressing and rapid large recovery on release indicate a "rubber-like" elastic behavior of the starch gel. Houwink (*10*) and others have suggested that rubber elasticity is associated with entropy changes in straightening, *vis-a-vis* curling, of the rubber molecules on stressing and release, respectively. Although the static modulus (2.9×10^5 dynes/cm.^{2}) is below that usually assigned to rubber-like materials (10^7-10^9 dynes/cm.^{2}), the lower modulus in the region of retarded elasticity (7.8×10^4 dynes/cm.^{2}) and the high viscosity (1.8×10^8 poises) are found in rubbery materials (see *1, 12*). However, intrachain forces probably are not strong as in rubber, and here the similar high elasticity may be due in part to the brush-heap arrangement of molecules which are not strongly attached in micelles, in part to configurational forces which condition the helical form of the starch molecule, and in part to entropy changes.

On application of a small stress, immediate deformation probably involves straightening of curled network chains between junction points as well as straining of large network regions. As the stress continues to act, some migration of unattached molecules may occur, and some of the weaker secondary bonds probably are broken. After recovery, these breaks and

molecular displacements will constitute part of the "flow" (permanent deformation) of the gel. (Note that this concept will negate the assumption of a Newtonian element, with laminar flow, in the mechanical model.)

With larger stresses, more breakage occurs, with some possibly even among the primary valence bonds. The increasing modulus (with applied stress) can involve (1) greater relative resistance of the more firmly organized micelles as the less firmly organized ones are disrupted, (2) reinforcement of these junction points (micelles), or (3) even the creation of new bonds due to the increasing parallelization of the starch molecules and their closer approach to each other during compression. The formation of new junctions would also tend to effect greater permanent deformation, hence "flow" (*1, 12, 13, 22, 23*).

An interesting clue to the molecular constitution of the gel is given by the "resilience," *i.e.*, ratio of recovered strain to the over-all strain (Table 2). These data show that the least relative permanent deformation occurs

TABLE 2

Stress and resilience (ratio of recovered strain to over-all strain) at 20-minute compression and recovery

Stress ($\times 10^3$ dynes/cm.^{2})	Resilience (%)
1.5	72
3.7	76
7.1	80
13.3	79
30.1	74
54.2	68

at stresses in the range of 7-13 x 10^3 dynes/cm.^{2} and that there is more relative permanent deformation at the stresses lower and higher than this range. Besides confirming the earlier diagnosis of molecular behavior at small and large stresses, a new outlook is added, *viz.*, that the ultimate strength of the majority of micellar bonds in this particular gel lies in the range of 7-13 x 10^3 dynes/cm.^{2}

Several other facets of behavior are in agreement with the above concept. In the curve, unrecovered deformation/stress, a dilatant effect is found up to a stress of about 10 x 10^3 dynes. Also in this intermediate stress region, the 18-second deformation shows a marked slope change (Fig. 7), indicating a change in the nature of the bonds being deformed. Similarly, the 18-second deformation at these stresses is about equal to the recovered deformation, whereas it is greater than the recovered deformation at larger stresses and less at smaller stresses.

Retardation of strain during compression perhaps may be understood, on a molecular level, in terms of (1) internal molecular friction in the starch gel, (2) continuous variation in the strength of bonds to be broken, and (3) formation of new junction points or reinforcement of older micelles in the network. During recovery, retardation of strain can be accounted for by (1) internal viscous and sliding friction, (2) the time involved for the straightened molecules to take random positions, and (3) possibly the retarding effect of the new bondings as the strained network resumes its original form.

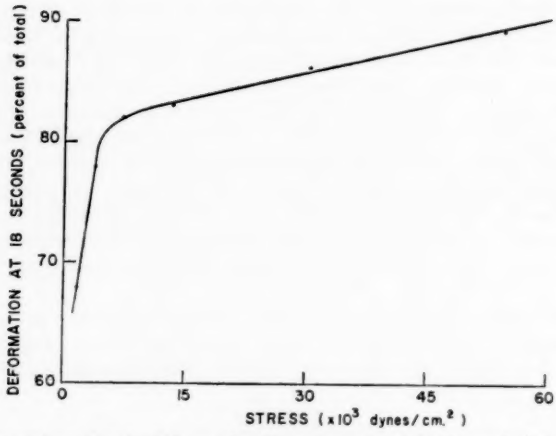


Figure 7. Proportionate deformation (as per cent of total) reached in 18 seconds of loading. Stress values are those at 18 seconds of loading.

The suggestion that new micellar units are formed during stress is founded on work with rubber and other polymers. Mark and Press (*13*) and others have suggested that the relationship, $\sigma = \mathbf{a} \ln t + \mathbf{K}$, denotes a second order mechanism and that this long-time logarithmic creep consists of (1) a crystallization term and (2) a series of retarded elastic elements. (Note that, in Figure 5, there are two linear regions in the curve of the stressed gel. In all probability, if any crystallization occurs, it is taking place in the time region of $4 \times 10^2 - 1.2 \times 10^3$ seconds.) Investigations on these polymers with X-rays (*6*), polarized light, and density determinations (*23*) (also see *1*) have indicated the validity of the crystallization phenomenon, particularly with increasing strain (therefore, also increasing applied stress).

A final factor, which has not been considered here, is the role of time of application of stress. Since the jelly candies in this experiment were all stressed for 20 minutes, the obvious question arises concerning the relationship of the time of stress to the relative amount of deformation and recovery. The consideration of a time relationship will be the concern of a subsequent study.

Summary

Starch jelly candies, at 24-hour aging, were put under compressive stress for 20 minutes under various loads and then allowed to recover for 20 minutes. Strain measurements were recorded continuously throughout loading and recovery. Some rubber-like behavior was noted, and a simplified mechanical model was suggested to explain the gross aspects of the strain-time curves. This model consisted basically of a Maxwell unit (elastic and viscous elements in series) connected with a modified Kelvin unit (variable elastic and thixotropic elements in parallel).

A molecular explanation of behavior assumed a reticulate framework of starch molecules with hydrogen bondings at the junction points, in which

are dispersed, in a brush-heap arrangement, free or less strongly-bonded starch molecules. Behavior under stress and recovery could be explained in terms of such organization with the additional suggestion of a crystallization effect (parallelization of starch molecules to form new micelles or stronger old micelles), with increased amount of stress.

LITERATURE CITED

1. Alfrey, T., Jr. *Mechanical Behavior of High Polymers.* 1948, Interscience Publ., Inc., New York, N. Y.
2. Chichester, C. O., and Sterling, C. Continuous recording of gel deformation in creep and recovery. *Food Research*, **21**, 397-401 (1956).
3. Cunningham, J. R., Hlynka, I., and Anderson, J. A. An improved relaxometer for viscoelastic substances applied to the study of wheat dough. *Canadian J. Technol.*, **31**, 98-108 (1953).
4. Davis, J. G. The rheology of cheese, butter and other milk products. (The measurement of "body" and "texture"). *J. Dairy Research*, **8**, 245-264 (1937).
5. Dempster, C. J., Hlynka, I., and Anderson, J. A. Extensograph studies of structural relaxation in bromated and unbromated doughs mixed in nitrogen. *Cereal Chem.*, **30**, 492-503 (1953).
6. Field, J. E. An X-ray study of the proportion of crystalline and amorphous components in stretched rubber. *J. Applied Physics*, **12**, 23-34 (1941).
7. Goodeve, C. F., and Whitfield, G. W. The measurement of thixotropy in absolute units. *Trans. Faraday Soc.*, **34**, 511-520 (1938).
8. Halton, P., and Scott Blair, G. W. A study of some physical properties of flour doughs in relation to their bread-making qualities. *Cereal Chem.*, **14**, 201-219 (1937).
9. Halton, P., and Scott Blair, G. W. The relationship between conditions governing rupture and flow in flour doughs. *J. Physical Chem.*, **40**, 811-819 (1936).
10. Houwink, R. *Elasticity, Plasticity and Structure of Matter.* 2nd ed., 1953, Harren Press, Washington, D. C.
11. Kuhn, W., Künzle, O., and Preissmann, A. Relaxationszeitspektrum, Elastizität und Viskosität von Kautschuk. I. *Helv. Chim. Acta*, **30**, 307-328 (1947).
12. Mark, H. Structure and mechanical behavior of high polymers. *Cold Spring Harbor Symp. on Quant. Biol.*, **9**, 204-209 (1941).
13. Mark, H., and Press, J. J. Elasticity, creep and recovery of acetate and viscose rayon yarns. *Rayon Textile Monthly*, **24**, 339-341, 405-407 (1943).
14. Meyer, K. H. The past and present of starch chemistry. *Experientia*, **8**, 405-420 (1952).
15. Press, J. J. Flow and recovery properties of viscose rayon yarn. *J. Applied Physics*, **14**, 224-233 (1943).
16. Reiner, M. *Deformation and Flow.* 1949, H. K. Lewis & Co., Ltd., London.
17. Röder, H. L. *Rheology of Suspensions. A Study of Dilatancy and Thixotropy.* 1939, Dissert., Univ. Utrecht.
18. Schofield, R. K., and Scott Blair, G. W. The relationship between viscosity, elasticity and plastic strength of soft materials as illustrated by some mechanical properties of flour doughs. I. *Proc. Roy. Soc. London*, **138** A, 707-718 (1932).
19. Schofield, R. K., and Scott Blair, G. W. The relationship between viscosity, elasticity and plastic strength of soft materials as illustrated by some mechanical properties of flour doughs. II. *Proc. Roy. Soc. London*, **139** A, 557-566 (1933).
20. Schofield, R. K., and Scott Blair, G. W. Über die grundlegenden mechanischen Eigenschaften des Mehlteiges. *Kolloid-Zeitschr.*, **79**, 148-154 (1937).
21. Scott Blair, G. W. *An Introduction to Industrial Rheology.* 1938, P. Blakiston's Son & Co., Inc. Philadelphia, Pa.
22. Treloar, L. R. G. Elastic recovery and plastic flow in raw rubber. *Trans. Faraday Soc.*, **36**, 538-549 (1940).
23. Treloar, L. R. G. Crystallisation phenomena in raw rubber. *Trans. Faraday Soc.*, **37**, 84-97 (1941).

CANNING EXPERIMENTS WITH NON-SPOREFORMING BACTERIA[a]

O. B. WILLIAMS
University of Texas, Austin

(Manuscript received March 24, 1956)

Ordinarily bacteriologists and technologists concerned with heat-processed foods have slight or no interest in the non-sporeforming organisms which may be present, either naturally or accidentally, on the food prior to processing. It is universally recognized that they are inferior in heat resistance to the least resistant spore, and consequently only under exceptional circumstances could such organisms be present in heat-processed food in a sound container. An example of such an exceptional circumstance would be the use of too low a filling temperature for a food of slow penetration which, because of high acidity, receives a boiling water process, or indeed may have no process beyond that afforded by air cooling. In such an instance one is dealing with acid foods, and the organisms concerned are either lactobacilli or yeasts. Furthermore, the presence of viable organisms is indicated by spoilage of the food. With the low acid and non-acid foods the presence of non-sporeforming bacteria in the heat-treated food is associated with, and is diagnostic of, spoilage due to a defective container. In this latter case a varied mixed flora among containers from the same lot will be found.

The work upon which this report is based, dealing with organisms of low resistance in a canned food, was prompted by a claim *(1)* that symptoms of gastroenteritis following the consumption of a stew made with canned oysters were due to a coccus which was isolated from washings from the can. The authors of the claim were of the opinion that the coccus had survived the process given the oysters, and had grown and produced an entertoxin without at the same time producing deteriorative changes which were organoleptically perceptible. The evidence for the presence of the coccus in the container prior to opening is not convincing since the culture was isolated from washings made some hours after the empty can had been discarded. However, it was decided to establish experimentally the fate of non-sporeforming bacteria introduced in large numbers into oysters canned under commercial conditions, and given different heat treatments. The present report summarizes the results which were obtained.

MATERIALS AND METHODS

Two organisms were used for experimentally inoculated packs. One was the coccus isolated by Majors, Scherago, and Weaver *(1)*, hereafter designated as Ky, which was supplied us through the courtesy of Dr. Scherago. The other was a known enterotoxin-producing strain of *Staphylococcus aureus* from the collection of Dr. G. M. Dack, and

[a] The work reported was done in the Research Laboratory of the National Canners Association, Washington, while the author was employed there. It is being published at this time in response to a recommendation made by one familiar with the work, who suggested that the data had sufficient general interest to justify being recorded.

identified by the number, 161. The ability of each of these organisms to grow in oyster brine was first established. Cultures on tryptone agar were washed down with 1% saline solution, shaken with sand to break up clumps, and inoculated into cans of oysters purchased on the open market. Counts on the brine were made at the start, and at intervals, by dilution-plating following shaking with sand of a diluted aliquot. The results of tests at 37° C. (98.6° F.) and at room temperature are given in the tabulation.

TABLE 1

Growth in oyster brine of organisms used in experimental packs

Time of incubation	Organism	Count per ml. of brine	
		37° C. (98.6° F.)	Room temp.
Start	161	1,200,000	860,000
	Ky	26,000	1,500
24 hours	161	44,000,000	43,000,000
	Ky	11,500,000	200,000
1 week	161	25,000,000	300,000,000
	Ky	3,400,000	5,500,000

The suspensions used to obtain the data given in Table 1 were held at room temperature and quantitative counts were made at intervals to determine whether the suspensions for inoculation purposes could be prepared in advance of the experimental pack without a significant decline in numbers before use. The count after 3 weeks was approximately one-half that at the start, from which it was concluded that the inoculum could safely be prepared some days in advance. The inoculum used consisted of 1 ml. of a suspension planted at the center of an oyster with a hypodermic syringe. The inoculated oyster was placed near the center of the can. The method of inoculation and the placing of the inoculated oyster in the can were done to give as favorable conditions for survival as possible. The suspensions used were counted two days after the pack was made with the following results: Number 161—5,500,000 per ml.; Ky—3,000,000 per ml. It may be assumed that the number of organisms inoculated is considerably in excess of what may be expected to be present under natural conditions.

The oysters were from Louisiana beds and were prepared for canning by steaming for from 6 to 12 minutes at 12 pounds' steam pressure, then shucked and flumed to a wash tank from which they were placed on trays to drain before filling. The fill was 4½ ounces in 211 × 400 (No. 1) cans. Brine of such strength that each can received 40 grains of salt was added at a temperature of 130° F. (54° C.). This salt concentration gives a percentage strength of about 1.6%. The sealing temperature was 110° F.

The cans included in the experimental packs were diverted from the canning line and differed from the regular run only in the inoculum and in the final heat treatment. Twelve cans for each variable for each organism were included with 12 uninoculated cans as a control.

The processes for one experimental lot were as follows: none, 10 minutes at 212° F. (100° C.), 5 minutes at 240° F. (116° C.), and 10 at 240° F. (116° C.). A second lot, held one hour after sealing before being heated, included 12 uninoculated cans processed for 5 minutes, 12 uninoculated cans processed for 10 minutes, 12 cans inoculated with Ky and 12 inoculated with No. 161, processed for 10 minutes, all at 240° F. (116° C.). No one of the experimental lots received as severe a cook as the commercial lots, which were processed for 18 minutes at 240° F. (116° C.) at the time.

The packs were shipped to the Washington laboratory for examination and culture.

RESULTS AND DISCUSSION

Upon receipt about a week after packing, all of the nonprocessed cans were hard swells. Quantitative counts on the brines ranged from 0.9×10^4 to 127×10^4. The Ky inoculated cans gave the lowest counts with the 161 inoculated and the control about equal, and some two to three times as great as the Ky lot. All showed a mixed flora microscopically, and upon

streaked plates and from broth tubes. The lot inoculated with No. 161 yielded an abundance of golden pigmented staphylococcus colonies. The pH ranged from 4.2 to 4.6. All cans given a process of any extent were flat.

Four cans of each of the other lots were examined in the same way at the time of receipt. The remaining 8 cans were divided with one lot being incubated at 37° C. (98.6° F.) and the other at room temperature. Cultures were made on 2 cans of each lot at 21 days and the final 2 cans were cultured after 31 days. With two exceptions all cans were microscopically and culturally sterile and in every respect normal. The pH of the brine was from 6.7 to 6.8. The two cans which were exceptions were from the lot inoculated with No. 161 and processed for 10 minutes at 212° F. (100° C.). These each yielded a pure culture of an aerobic spore-forming rod.

The results recorded were somewhat surprising in the low number of cans showing viable bacteria among those given the lower cooks. It would appear that heat resistant contamination is not common in oysters for canning. However, the commercial process, which is designed to protect the consumer against chance contamination with resistant spores of health significance, cannot safely be lowered because of these data developed for a special purpose.

The results speak conclusively against the possibility of a non-spore-forming organism surviving the commercial process.

SUMMARY

Two different coccus forms, one unidentified and one a known enterotoxin producing *Staphylococcus aureus*, were found to grow in oyster brine. Large numbers of each were inoculated into oysters in 211 × 400 cans. Non-processed cans became hard swells with a mixed flora, but no can processed as much as 10 minutes at 212° F. (100° C.) showed the survival of cocci, and only two such cans showed surviving spores.

LITERATURE CITED

1. Majors, Paul, Scherago, M., and Weaver, R. H. Staphylococcus food poisoning from canned oysters. *J. Bact.*, **35**, 72 (1938) and personal communication.

DIFCO

Media for

MYCOLOGY

DIAGNOSTIC

. . . for the isolation, identification and cultivation of pathogenic fungi. These media are also widely used in phytopathological studies. Several are neutral in reaction, giving optimum conditions for growth of a variety of fungi. The following may be prepared as selective media by the adjustment of reaction, addition of antibiotics or other agents:

Bacto-Brain Heart Infusion Agar
Bacto-Sabouraud Dextrose Agar
Bacto-Sabouraud Maltose Agar
Bacto-Littman Oxgall Agar
Bacto-Mycological Agar
Bacto-Mycological Broth
Bacto-Corn Meal Agar
Bacto-Corn Meal Agar with Dextrose
Bacto-Bean Pod Agar
Bacto-Prune Agar
Bacto-Lima Bean Agar

CONTROL

. . . for sanitary and sterility procedures as well as for general use in mycological procedures:

Bacto-Sabouraud Maltose Broth
Bacto-Sabouraud Liquid Medium
Bacto-Malt Extract
Bacto-Malt Agar
Bacto-Neurospora Culture Agar
Bacto-Potato Dextrose Agar
Bacto-Mildew Test Medium
Bacto-W.L. Nutrient Medium
Bacto-W.L. Differential Medium

CLASSIFICATION

. . . and nutritional studies of fungi:

Bacto-Yeast Morphology Agar
Bacto-Yeast Carbon Base
Bacto-Yeast Nitrogen Base
Bacto-Czapek Dox Broth
Bacto-Czapek Solution Agar
Bacto-Vitamin Free Yeast Base

THE DIFCO MANUAL, *NINTH EDITION,*
including descriptions of these media and their use,
is available on request.

DIFCO LABORATORIES
DETROIT 1, MICHIGAN